



Contribution of PDZD8 to Stabilization of the Human Immunodeficiency Virus (HIV-1) Capsid

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**Contribution of PDZD8 to Stabilization of the Human Immunodeficiency
Virus (HIV-1) Capsid**

A dissertation presented

by

Charles Alexander Guth

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Virology

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**Contribution of PDZD8 to Stabilization of the Human Immunodeficiency
Virus (HIV-1) Capsid**

Abstract

Following human immunodeficiency virus (HIV-1) entry into the host cell, the viral capsid gradually disassembles in a process called uncoating. A proper rate of uncoating is important for reverse transcription of the HIV-1 genome. Host restriction factors such as TRIM5 α and TRIMCyp bind retroviral capsids and cause premature disassembly, leading to blocks in reverse transcription. Other host factors, such as cyclophilin A, stabilize the HIV-1 capsid and are required for efficient infection in some cell types. To identify additional cellular factors that alter retroviral core uncoating, we developed a novel *in vitro* assay of HIV-1 capsid stability. Using this assay, we have shown that a factor in the cytoplasm of cells from multiple vertebrate species slows the spontaneous disassembly of HIV-1 capsid-nucleocapsid (CA-NC) complexes *in vitro*. We identified the PDZ-Domain-containing protein 8 (PDZD8) as a critical component of the capsid-stabilizing activity in the cytoplasmic extracts. PDZD8 has been previously reported to bind the HIV-1 Gag polyprotein and to make a positive contribution to the efficiency of HIV-1 infection. PDZD8 knockdown accelerated the disassembly of HIV-1 capsids in infected cells, resulting in decreased reverse transcription. The PDZD8 coiled-coil domain is sufficient for HIV-1 capsid binding, but other parts of the protein, including the PDZ domain, are apparently required for stabilizing the capsid and supporting HIV-1 infection. In summary, PDZD8 interacts with and stabilizes the HIV-1 capsid and thus represents a potentially targetable host cofactor for HIV-1 infection.

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ATTRIBUTION OF PRESENTED FIGURES

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Chapter One

Introduction

1.1.0.0 Introduction to HIV

Human immunodeficiency virus (HIV) is the pathogen that causes acquired immunodeficiency syndrome (AIDS) in infected persons. As of 2011, 34 million individuals were infected with HIV, including ~0.8% of the world's population between the ages of 15 and 49 (312). These numbers continue to rise with 2.5 million new infections annually (312). Without treatment, HIV infection progresses to AIDS and death. Despite advancements in the treatment of HIV infection, ~1.7 million infected persons die from AIDS-related causes worldwide each year (312). Beyond mortality, the AIDS pandemic has far reaching socio-economic impacts, particularly in developing nations. Many of these nations face HIV infection rates that are amongst the world's highest. It is estimated that there are currently 16 million children orphaned worldwide due to the effects of AIDS (312). The costs of AIDS and its prevention are substantial. Domestic spending by low and middle-income countries on AIDS-related health costs was greater than \$8 billion in 2011 (312). In the most affected nations, the costs of AIDS infection represented >60% of total health spending (9). International aid for AIDS related expenses, ~\$9.5 billion annually, is greater than that for any other disease (313). In high-income countries, the lifetime cost of treatment for each newly infected person is estimated to be between \$350,000-1.3 million (278). Since its global emergence in the late 20th century, HIV infection has been and continues to be among the greatest crises in global public health.

AIDS was first described in 1981 in response to a spike in the prevalence of several rare opportunistic infections including Kaposi's sarcoma and *Pneumocystis carinii* pneumonia (57). Affected individuals were found to have attenuated cell-mediated immunity responses due to abnormally low levels of circulating of CD4⁺ T cells (132). Early theories of AIDS induction included allergy and drug reaction (128, 221), but study of AIDS transmission suggested the

etiological agent was viral (104). In 1983, the relationship between AIDS and retroviral infection was first reported, and in 1984 it was demonstrated conclusively that the infectious agent responsible for AIDS was the novel retrovirus HIV type I (HIV-1) (then identified as lymphadenopathy-associated virus or human T-cell leukemia virus type III) (45, 61, 93, 117, 164, 276). A second HIV type (HIV-2) was identified shortly thereafter (68).

In the years following the identification of HIV as the causative agent of AIDS, diagnoses of HIV worldwide rose precipitously, becoming a global pandemic with ~60 million individuals infected since 1984 (312). Compounding the difficulty of public health responses, HIV infection is concentrated in low and middle-income nations where greater than 90% of infected persons reside. HIV infection reaches its greatest prevalence in sub-Saharan Africa with adult infection rate surpassing 25% in the most-affected nations (312).

Despite significant efforts, no effective vaccine for HIV exists. However, over the past 15 years, the development of HIV small molecule antivirals and the expansion of public health initiatives have slowed the spread of HIV. Worldwide infection rates have decreased by ~30% from peak levels in the late 1990s (312). The introduction of antiretroviral drug therapy targeting HIV has served to not only decrease the potential of treated persons to pass on the virus to uninfected individuals but also to dramatically change the prognosis for infected individuals, slowing and reversing the progression of AIDS in most treated patients (16).

The continued investigation of HIV molecular biology is an important component of future work intended to reverse the spread of the virus and treat infected individuals. Here, I present evidence from investigations of HIV-1 capsid uncoating with a focus on a cellular cofactor, PDZD8, which contributes to the regulation of that process.

1.1.1.0 Molecular biology of HIV

The virions of all retroviruses, including HIV, carry two copies of a positive-sense RNA genome. Upon infection, this RNA genome is reverse transcribed into a cDNA genome, which is then integrated into host chromosomal DNA (150). This integrated genome is called the provirus. The viral genome is ~9700 nucleotides in length and contains nine open reading frames (ORFs) coding the virus's 15 structural and regulatory proteins, as well as several conserved RNA elements critical to gene expression, packaging, and integration (107). A brief introduction to these genes and RNA features is given below.

1.1.1.1 Genes and Protein Products

gag- The *gag* gene encodes the 55-kDa precursor polyprotein, group specific antigen (Pr55 Gag). Upon maturation, Pr55 Gag is processed by the viral protease into the proteins matrix, capsid, nucleocapsid, and p6. Processing also results in the creation of two small spacer proteins (P1 and P2). Gag and its component proteins described below are responsible for the organization and assembly of new virions.

Matrix (MA)- The 17-kDa N-terminal component of Pr55, MA is a protein critical to the association of Gag with the cellular membrane prior to budding. The first 50 amino acids of MA, including an N-terminal myristate group, mediate the association of Gag with cellular membranes (242, 274). MA also contributes to the recruitment of the viral envelope to the sites of virion budding (69).

Capsid (CA, p24)- The 24-kDa CA protein forms the characteristic conical protein capsid of the mature virion's core. Intermolecular associations by the CA region of Gag

drive the protein's multimerization and the condensation of the intact viral capsids of immature virions (124). Upon cleavage of Gag by the viral protease, the CA protein forms the mature viral capsid, the exterior of the genome-containing viral core. Following entry, the intermolecular self-association of CA as well as the interaction of CA with additional cellular cofactors are determinants of the kinetics of viral uncoating. The regulation of this process has important implications for reverse transcription and nuclear import (102, 186, 220).

Nucleocapsid (NC)- The 7-kDa NC protein, which contains two nucleotide binding zinc-finger domains, targets the viral RNA genome to the assembling virion (74). Binding of the genome by NC may also serve to protect the RNA from cellular nucleases.

P6- The 6-kDa P6 domain of Gag regulates the late-stage separation of virions from the cell surface. P6 contains two late assembly (L-) domains that recruit cellular cofactors necessary for budding (231). Additionally, P6 is responsible for mediating incorporation of the viral protein Vpr into the virion (182).

pol: In 5-10% of translation events, a ribosomal frame shift results in the production of the precursor polyprotein Gag-Pol (P160) (41). Cleavage of the Pol component of P160 results in the viral protease, the reverse transcriptase, and integrase.

Viral Protease (PR)- The 15-kDa viral protease is responsible for the cleavage of all individual proteins from both Gag and Gag-Pol including PR itself. PR is functional as a dimer. For this reason, the initiation of cleavage does not occur until accumulation of

Gag-Pol results in concentrations sufficiently high to drive PR dimerization (185). The completion of Gag cleavage by PR results in the formation of mature virions.

Reverse Transcriptase (RT)- Composed of two subunits 51-kDa and 66-kDa in size, RT catalyzes the RNA-dependent and DNA-dependent polymerization reactions of reverse transcription. Reverse transcription of the genome by RT results in the formation of a cDNA copy of the RNA viral genome, which is subsequently integrated into the host cell genome as the provirus. RT also contains the RNaseH domain responsible for degradation of the RNA-DNA hybrid intermediates, a requirement for efficient reverse transcription processivity (150).

Integrase (IN)- The 32-kDa IN protein catalyzes the integration of the viral cDNA genome into the host chromosome following reverse transcription and nuclear import. Integrase, which functions as a tetramer, both processes the cDNA genome and ligates it to the host chromosome during viral integration (71).

vif- The *vif* gene codes one protein, the viral infectivity factor (Vif). 23-kDa Vif enhances infectivity by targeting the cellular restriction factor APOBEC3G for degradation by the proteasome (286). In the absence of VIF, APOBEC3G induces hypermutation of the viral genome, significantly decreasing infectivity (217).

vpr- The *vpr* gene encodes the 14-kDa viral protein R (Vpr) that has a minor role in the regulation of the import of the HIV-1 preintegration complex into the nucleus (141). Vpr has

additional roles in cell cycle arrest, suppression of the immune response and induction of cellular differentiation.

tat- The *tat* gene encodes the transactivator of HIV-transcription (Tat). Tat protein binds the trans-activating response element present in the 5' ends of nascent viral transcripts, enhancing the rate of transcript elongation. Tat binding enhances transcription of both viral genomes and all viral genes, including *tat*, resulting in a positive feedback loop (165, 292). This feedback loop drives the transition of early-stage viral gene expression to late-stage gene expression in infected cells.

rev- The *rev* gene encodes the regulator of expression of virion proteins (Rev) factor. Rev binds a response element in viral RNAs permitting the export from the nucleus of both full-length RNA genomes as well as mRNAs encoding Gag, Pol, the viral envelope, Vif, and Vpr (213). Rev recruits cellular export factor Crm1, which mediates the export of Rev-bound viral RNAs through the nuclear pore (31).

vpu- The *vpu* gene encodes the viral protein u (Vpu) factor, which induces degradation of cellular CD4 and permits efficient release of budding virions. Vpu mediates the binding of cellular CD4 to E3 ubiquitin ligase complex, inducing degradation of the CD4 protein in the endoplasmic reticulum (326). Degradation of CD4 is thought to contribute to the proper incorporation of the viral envelope, a CD4-binding protein, into nascent viral particles. Vpu also down regulates the surface expression of the cellular restriction factor tetherin. In the absence of

Vpu, tetherin prevents newly budded virions from dispersing from the host cells. Vpu targeting of tetherin permits efficient virion release (236, 237).

env- The *env* gene encodes the viral envelope (Env or gp160). Cleavage of Env by cellular proteases creates gp120, the viral docking glycoprotein and gp41, the transmembrane glycoprotein. These two proteins remain associated after cleavage and form trimers on the virion surface. gp120 recognizes the receptor CD4 and co-receptors of HIV. Following binding to target cells, gp41 catalyzes the fusion of viral and cellular membranes, facilitating viral entry (325).

nef- The *nef* gene encodes the negative effector protein (Nef). Nef enhances viral infectivity by altering the cellular activation state of target T-cells and down-modulating surface expression of CD4 and MHC in infected cells (191).

1.1.1.2 Notable DNA/RNA features

5' and 3' LTR- The DNA proviruses of retroviruses are characteristically flanked by identical sequences, called the long terminal repeats (LTRs). The HIV LTR is ~640 nucleotides in length and, like all retroviral LTRs, is composed of 3 regions—5' unique (U5), repeat (R), and 3' unique (U3). The U3 region contains the viral transcription promoter and enhancer regions. The R region is necessary for the strand transfer step of reverse transcription of the RNA genome. The viral RNA genome does not contain two complete copies of the LTR but has short repeat (R) regions at both ends. Full LTRs are generated in the cDNA genome during the process of reverse transcription (107).

TAR hairpin- The transactivation-response (TAR) region is a stem loop structure formed in 5' end of the RNA transcript, 3' to the site of proviral transcription initiation. The viral protein Tat binds TAR, recruiting cell factors (*e.g.* cyclin T) which significantly enhance the rate of viral transcript elongation, leading to higher transcript levels (272).

RRE- The Rev response element (RRE) is a 351-nt elongated stem loop structure to which the viral protein Rev binds with high affinity. Binding of Rev at this site permits the rapid export of unspliced or incompletely spliced viral mRNA (213).

Packaging signal (Ψ)- The viral RNA packaging signal, Ψ , is approximately 150 nt in length and forms four stem-loop structures. The NC domain of uncleaved Gag specifically binds this sequence, leading to recruitment of viral transcripts to the site of virion assembly. Splicing of viral transcripts results in the loss of Ψ ; consequently, only unspliced transcripts, which can function as the genome of new virions, are specifically bound by Gag (74).

Gag-Pol frameshift- Transcription of Gag-Pol requires a -1 frame shift during transcription prior to the *gag* gene stop codon. This frameshift occurs in ~1 of 20 transcriptions of *gag* due to two cis acting RNA elements ~200 nt upstream of the stop codon—a six uracil sequence and a stem loop pseudoknot. These elements induce a pause in transcription, sometimes resulting in the frameshift necessary for production of the Gag-Pol polyprotein (155).

1.1.2.0 Lifecycle of HIV

To infect target cells, HIV makes use of many cellular proteins, called cofactors, which perform roles necessary for the completion of the viral lifecycle. In contrast to these positive cofactors, other target cellular proteins, restriction factors, inhibit viral infectivity. The research described here was undertaken with the objective of identifying novel cellular cofactors and restriction factors that act during the pre-integration steps of HIV-1 infection. In later chapters of this dissertation, we will present evidence that the cellular protein PDZD8 enhances viral infection by contributing to the regulation of viral capsid uncoating following entry. Here, we present a brief outline of the entire viral life cycle, noting some of the cellular cofactors and restriction factors that are involved in each step.

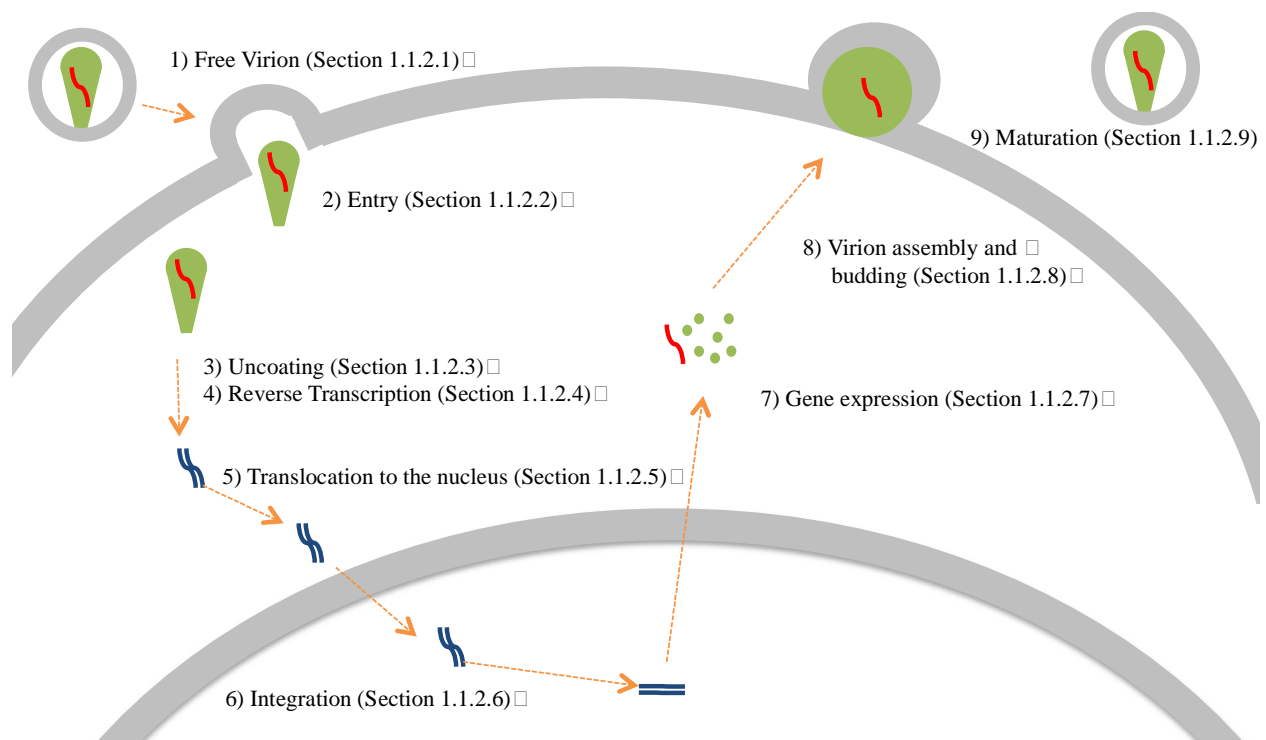


Figure 1.1 The lifecycle of HIV-1.

1.1.2.1 Free virion

The mature HIV-1 virion is a spherical double-membrane bound particle ~80-130 nm in diameter (249). The lipid membrane of the virion is derived from the producer cell. However, relative to concentrations in whole producer cell membranes, viral membranes are enriched in cholesterol, phosphatidyl ethanol, sphingomyelin, and phosphatidyl serine (7, 8). These lipid concentrations are consistent with the detergent-resistant lipid rafts of the producer cells from which nascent virions most commonly bud (44). Integrated into the membrane are glycosylated ‘spikes’ composed of trimers of the viral enveloped proteins gp120 and gp41. The average virion features ~7-14 trimers which extend 10 nm for the virion surface (354).

Contained within this viral membrane is the protein-bound viral core particle that is formed upon cleavage of Gag by the viral protease at the time of virion release. Following Gag cleavage, the MA protein forms a discontinuous shell associated with the inner leaflet of the membrane. This shell is organized by a hexameric array of MA trimers (327). The CA subunit condenses into the characteristic conical capsid composed of ~250 hexameric rings and 12 pentamers of CA protein (257, 349). This capsid and its contents are also known as the viral core. Within the capsid are two copies of the RNA genome (248). The two copies are both 5’ capped and 3’ polyadenylated as well non-covalently linked through a homodimerization interaction between “kissing loop” sequences in the 5’ untranslated regions of each RNA molecule. In the mature virion, the cleaved NC protein coats the genome and contributes to its condensation into a more tightly packed arrangement within the capsid shell. The viral core also contains the component proteins of the P160 Gag-Pol precursor including RT and IN, which are required for post-entry events. Limited levels of the accessory proteins Vpr, Vif, and Nef have also been reported to be present in the viral core. Multiple cellular factors are specifically

incorporated into the virion as well. These include cytokine receptors integrated in the viral membrane, tRNA molecules required for reverse transcription in the viral core, and the host cell restriction factor APOBEC3G (245).

1.1.2.2 Entry

Viral infection begins with the binding of the virion to the target cell and its subsequent entry into that cell. This process is accomplished primarily through actions of the viral Env proteins. On mature virions, the Env protein has been cleaved into the gp120 and transmembrane gp41 subunits that remain non-covalently linked and assembled as trimeric spikes which extend from the viral surface (111, 112).

Adhesion of the virion to the target cell may occur from either recognition by Env of specific cellular surface molecules including pattern recognition receptor and $\alpha 4\beta 7$ integrin or non-specific affinity for negatively charged cell-surface heparin cell-surface proteoglycans (15, 66, 126, 211, 223, 244, 275). Adhesion molecules incorporated into the HIV-1 virions and cognate receptors on the target cell assist virion-cell attachment (307). The association of Env and these attachment factors bring the spike protein trimers into close proximity with the cellular receptor, CD4, located on the target cell's surface.

Subsequent to cellular attachment, gp120 binds the primary HIV-1 receptor CD4. Binding of CD4 induces significant rearrangement of the Env trimer, rearranging the V1, V2, and V3 loops of gp120 and exposing the co-receptor binding site (189, 308, 328). After binding CD4, gp120 is capable of binding a co-receptor molecule (typically CCR5 or CXCR4) (63, 81, 89, 100). Binding of the co-receptor induces further rearrangement of the spike protein, exposing the fusion peptide of gp41 which enters the target cell membrane (60). The insertion of

the gp41 fusion peptide into the cellular membrane tethers the viral spike to the target cells and stimulates the transition of gp41 into its fusogenic confirmation, bringing the viral and cellular membrane into very close proximity. This results in the formation of a membrane fusion pore (60, 322). This pore is the means by which the viral core enters the target cell (224).

1.1.2.3 Uncoating

Following fusion of the viral and cellular membranes, the HIV-1 core is released into the cellular cytoplasm. There, the capsid undergoes a series of morphological changes which result in the disassociation of CA from the pre-integration complex at a point prior to the completion of genome translocation into the nucleus (12). This series of actions are collectively called uncoating. The specific nature of the processes that comprise uncoating as well as the location and time post-infection at which these processes take place remain areas of ongoing investigation.

Though there remain many outstanding questions regarding the process of uncoating, there is ample evidence that the regulation of uncoating kinetics is of critical importance to viral infectivity (102, 299). The mature viral capsid is a metastable structure maintained by relatively weak CA-CA intermolecular interactions. Perturbation of these interactions by alteration of CA residues has proven to have significant consequences during retroviral infection. Premature disassembly of the core significantly diminishes viral infectivity (299). Similarly, *gag* mutations that contribute to slower than typical uncoating also result in inhibition of infectivity (102).

Host factors also play a role in regulation of the viral uncoating rate. The host cofactor cyclophilin A enhances HIV-1 infectivity in some target cells by promoting stability of the core (202). Additionally, as yet unidentified cellular cofactors have been proposed to play a role in

stimulating active uncoating of the viral capsid (17). Conversely, the cellular factor TRIM5 α and cytoplasmically localized CPSF6 can restrict viral infectivity by respectively enhancing and slowing the rate of viral uncoating (78, 113, 298, 299).

The process of uncoating is closely linked to that of reverse transcription. Initial models of uncoating posited that the disassembly of the intact viral capsid might be required to ‘release’ the viral genome into the target cell cytoplasm and initiate reverse transcription (12). More recent evidence has suggested the opposite—that capsid stability is necessary for proper production of reverse transcripts. Premature uncoating of the capsid due to *gag* mutations or capsid-destabilizing restriction factors inhibits viral infection prior to the completion of reverse transcription (102, 299). Though the exact role of CA in reverse transcription is not known, it has been hypothesized that the capsid may act as a scaffold for reverse transcription complexes, or act to protect those complexes from inhibitory cellular restriction factors (12). Additionally, there is mounting evidence that reverse transcription may contribute to the regulation of uncoating. Inhibition of reverse transcription has been reported to slow uncoating (11, 14, 152).

In subsequent chapters, we will present information about a novel assay of capsid stability as well as evidence that a cellular cofactor, PDZD8, enhances HIV-1 infectivity by slowing core uncoating. For this reason, section 2 of this chapter addresses the process of uncoating and associated cellular factors in greater detail.

1.1.2.4 Reverse transcription

While in the target cell cytoplasm, the RNA genome of the virus undergoes reverse transcription, a multistep process that produces a cDNA copy of the genome. This cDNA genome will be subsequently integrated into a host chromosome as a provirus following nuclear

import. The process of reverse transcription is catalyzed by the viral RT protein, but also requires multiple cellular host factors for both initiation and completion (150).

The HIV-1 RT molecule has two distinct components, both required for completion of reverse transcription: first, a DNA polymerase capable of using either DNA or RNA as template; second, an RNaseH component that degrades RNA from RNA-DNA hybrids (338). The polymerase of RT requires priming of the template RNA strand. During infection, this priming is performed by the binding of cellular tRNA^{Lys3}, which is packaged with the RNA genome in budding virions (20, 58). The tRNA primer binds the viral genome at a primer-binding site approximately 180 nucleotides from the 5' end of the genomic RNA adjacent to its U5 region (266, 269). The process of reverse transcription begins with reverse transcription of a minus-strand DNA copy of that first 180 nt region by RT (304). This first reverse transcription primarily takes place following viral entry, possibly driven by the ample concentration of free nucleotides there; though there is some evidence that limited reverse transcription of this region may occur within the virion itself prior to membrane fusion (151, 207, 309, 323, 353). This minus-strand DNA transcript creates an RNA-DNA hybrid over this 5' region of the RNA genome that is a target for degradation by the RNaseH domain of RT. After RNaseH digestion, the newly transcribed minus strand DNA binds a complementary sequence in the repeat (R) region of RNA genome's 3' end, becoming a primer for reverse transcription of the remaining RNA genome. As the reverse transcription of the genome continues, the resulting minus-strand DNA transcript forms an additional RNA-DNA hybrid with the template genome, targeting that genome for degradation by the RNaseH domain. One segment of the genome, a region near the 3' untranslated region, the polypurine tract, is resistant to RNaseH digestion. This preserved fragment of RNA serves as the primer for the synthesis of positive strand DNA by RT. When

this positive strand DNA synthesis reaches the 5' of the negative-strand template, including that region initially primed by the tRNA, that strand then transfers to the initial tRNA primer binding site on the 3' end of the negative strand DNA. There reverse transcription continues to synthesize a full-length cDNA positive strand (150).

In addition to RT and the RNA genome, the reverse transcription complexes (RTCs) have also been reported to contain viral proteins NC, IN, Vpr, MA and CA, which contribute to both reverse transcription and subsequent steps of infection (92, 136, 159, 179, 329, 344). Reverse transcription efficiency is enhanced by the presence of the nucleic-acid-binding protein NC, likely due to a genome-chaperoning function of that protein (197, 267). CA and IN association are required for subsequent steps, specifically nuclear import and cDNA integration (88, 194, 263, 333). CA may also play a role in reverse transcription as well, potentially as a scaffold to protect or organize RT and genomes (96). Much of reverse transcription occurs contemporaneously with viral capsid uncoating and the two processes appear functionally linked as well (11, 14, 70, 102, 152, 333). Inhibition of reverse transcription slows the initiation of some steps of uncoating, while deregulated uncoating can inhibit the production of reverse transcribed cDNA genomes (11, 14, 152, 299, 339).

1.1.2.5 Translocation of the HIV pre-integration complex into the nucleus

Following reverse transcription within the RTC, the viral cDNA remains associated with viral factors in a high-molecular-weight complex called the pre-integration complex (PIC). In order to deliver the cDNA to the host chromosome for integration, the PIC must enter the nucleus. Though some other retroviruses reach the chromosomes during cell division (271), evidence suggests that for HIV-1 infection, passage through the nuclear membrane is typically

required in both proliferating and non-proliferating cells (172). To accomplish this task, HIV makes use of multiple host factors to mediate passage through the nuclear pore complex (NPC) (10, 37, 40, 64, 172, 183, 198). This process can be divided into two parts—the transport of the RTC/PIC to the perinuclear space and the import of the PIC into the nucleus.

The specific mechanisms of RTC/PIC transport to the perinuclear space are incompletely understood, but preliminary evidence suggests this process may be dependent upon association of the RTC/PIC with the cell's cytoskeleton, specifically microtubules (MTs) (342). HIV-1 RTC/PICs have been shown to co-localize with microtubules and to concentrate at microtubule organizing centers (MTOC) (222). MTOCs are located in the perinuclear region of the cell. Long filamentous microtubules run from these centers to the periphery of the cell, suggesting a mechanism by which RTC/PICs travel along microtubules to reach MTOCs near the nuclear membrane in advance of translocation through the nuclear pore (2, 94). However, direct evidence of a necessary MT-dependent role in perinuclear localization has been mixed. Though intracellular injection of MT-targeting antibody inhibited perinuclear MTOC localization of RTC/PICs, MT depolymerizing molecules have had only minimal effect on HIV-1 infectivity (46, 222, 342). There may be multiple, redundant cytoskeletal-dependent mechanisms by which RTC/PICs are transported into proximity with the nuclear pore.

The translocation of the PIC into the nucleus occurs through the nuclear pore complex and is dependent on multiple viral and cellular cofactors (48, 49, 79, 198, 317). Viral factors including MA, IN, Vpr, and specific viral cDNA structures have been hypothesized to mediate nuclear import; however, no specific nuclear import signal has been conclusively demonstrated to be necessary for PIC import (103, 109, 110, 116, 141, 205, 215). Though uncoating of the capsid results in the disassociation of most or all of the CA protein from the viral cDNA prior to

completion of PIC nuclear import, it appears the CA protein plays a critical role in the completion of the process (186, 335).

Studies of mutant and chimeric retroviruses indicate that HIV-1 CA is the key determinant of infection in cell-cycle arrested target cells—which require import through the nuclear membrane (335). The role of CA in regulating nuclear import has been proposed to be twofold. First, CA-CA interactions are a primary determinant of uncoating (as described above and in greater detail in section 1.2.3). Because intact capsid is too large to import through the nuclear pore, factors that inhibit uncoating, including CA alteration, may potentially inhibit the import of the PIC. Second, substantial evidence exists that CA may play a role in enhancing import by mediating recruitment of the RTC/PIC to cellular host factors including Nup153, TNPO3, and Nup358 (40, 183, 186, 220, 279). The HIV PIC uses these factors, as well as other components of the nuclear pore, to access the interior of the target cell nucleus.

The process of nuclear import also appears to have implications regarding the subsequent process of cDNA integration. Inhibition of CA interaction with host cell import factors Nup153, Nup358, or TNPO3 by mutation or siRNA knockdown results in alteration of the integration sites of the provirus (181, 241, 279). Integration under these altered conditions occurs less frequently in gene-dense regions than in typical infection.

1.1.2.6 Integration

Integration of the viral cDNA into the host chromosome to form a provirus is a defining characteristic of retroviruses. This integration is required for subsequent transcription of viral genes and replication of the viral genome. Integration is primarily mediated by the viral protein IN, but multiple cellular factors also contribute to the process (71, 311).

The process of cDNA integration begins with the removal of two nucleotides from the 3' end of each DNA strand (called 3' processing) leaving two recessed strand ends terminated in hydroxyl groups. These 3' hydroxyl groups then attack a pair of phosphodiester bonds on target host genomic DNA strands. In HIV-1 infections, the target bonds are separated by five nucleotides (156). This attack produces a staggered cut of the genomic DNA and joins the 5' of the target DNA to 3' of the viral cDNA in an intermediate product. Finally, the unpaired 5' ends of the viral cDNA are removed and DNA-repair mechanisms fill the gap between the genomic DNA and the viral 5' ends, ligating the two strands and completing integration (71).

The selection of genomic sites of integration is not highly sequence-specific, though weak sequence preference has been observed (25, 148, 296, 331). Beyond sequence, integration appears to occur preferentially at sites with sharp bends or other distortion as well as at sites undergoing active transcription (21, 32, 228, 262, 280, 320, 330). The selection of actively transcribed human genes for integration may increase the efficiency of proviral transcription. This active-gene targeting is mediated by the viral IN protein, the cellular IN-binding protein lens epithelium-derived growth factor (LEDGF), and mechanisms of nuclear import as discussed above in Section 1.2.5 of this chapter.

Integration requires the involvement of cellular factors that contribute at multiple stages within the process of integration. Analysis of viral PIC composition has identified several cellular proteins that interact with the complexes and may be required for efficient integration including, barrier-to-autointegration factor (BAF), high-mobility-group proteins (HMGs), and LEDGF (67, 125, 206, 289, 300). Of these, the role of LEDGF is best understood. LEDGF is a critical cofactor of HIV-1 integration which binds the IN molecule and tethers the PIC to host chromatin (67). Host factors are required too for completion of integration following the 3'-

hydroxyl attack. The removal of unpaired 5' viral nucleotides and the ligation of the 5' end of the viral cDNA into the target genomic DNA is accomplished by the cell's DNA double-strand break repair machinery, though the specific components involved remain controversial (19, 75, 199).

1.1.2.7 Gene expression

The integrated HIV-1 provirus serves as the template for the subsequent transcription of viral genes and RNA genomes. Transcription of viral genes occurs in two stages. Early-stage gene expression produces predominantly multiply spliced transcripts encoding the viral proteins Rev, Tat, and Nef. In late-stage gene expression, transcription levels are increased and many transcripts are exported from the nucleus before terminal splicing, creating mRNA for all viral proteins as well as full-length viral RNA genomes. This transition is regulated by the viral factors Tat and Rev, which work in concert with elements of the cellular transcription and nuclear transport machinery (169, 291).

Transcription of the provirus is driven by the U3 promoter region in the 5' LTR (137, 160). The LTR promoter is an efficient initiator of transcription; however, in the absence of viral Tat, the cellular negative elongation factor (NELF) binds the provirus and restricts transcript elongation, severely limiting transcription efficiency (36, 154, 165, 178). Consequently, in early-stage gene expression, viral transcript levels are low. During this early stage, viral transcripts are spliced multiple times before export into the cytoplasm, producing mRNAs encoding for the Tat and Rev proteins that drive the transition into late-stage gene expression. The accessory protein Nef is also produced during early-stage gene expression.

Expression of the viral transactivator protein Tat drives a significant increase in transcription levels (292). Tat protein binds the TAR RNA-loop, located just downstream of the viral promoter, recruiting the cellular positive-acting elongation factor b (P-TEFb) (86, 87, 145, 146, 355). Tat binding and recruitment of P-TEFb initiates a series of phosphorylations of additional cellular factors that regulate the rate of viral transcription. These factors include RNA polymerase II and DRB sensitivity-inducing factor (154, 178). The phosphorylation events lead to a substantially enhanced level of viral transcript elongation and the more rapid production of viral transcripts.

The capability of Tat to stimulate transcription of viral mRNAs, including Tat-encoding mRNA, creates a positive feedback loop in viral transcription. Conditions that maintain low levels of Tat expression delay the eventual high-transcription levels of the late-stage gene expression. This delayed Tat-mediated positive feedback is known as latency (168, 290). Infected cells may persist, producing low levels of viral mRNA until Tat levels reach a threshold necessary to drive the feedback loop. When Tat levels pass that threshold, provirus transcription significantly increases and drives the cell into late-stage gene expression.

Late-stage gene expression is defined not only by the level of mRNA transcription, but also by the type of RNA exported from the nucleus (177). Terminally spliced viral RNAs encode only the Tat, Rev, and Nef proteins. Ordinarily, cellular factors degrade RNA molecules that are not fully spliced. The viral Rev protein exports unspliced or non-terminally spliced transcripts before degradation can occur. These incompletely spliced RNAs both encode viral proteins as well as serve as the RNA genome in new virions (213).

The Rev protein, which is produced during early-stage gene expression, binds the RNA structural element RRE present in incompletely spliced viral transcripts (213, 291). On the RRE-

containing transcripts, multiple molecules of Rev oligomerize and recruit the cellular cofactor exportin 1 (XPO1) through a C-terminal nuclear export sequence (212, 214, 347). XPO1 is a nuclear export factor and mediates the transport of these sequences into the cytoplasm through the nuclear pore (76).

Cytoplasmic viral RNAs serve as the template for translation of viral proteins as well the genomes packaged into virions (177, 255). Unspliced mRNA serves as the template for both the Gag and Gag-Pol polyproteins (155). However, translation of Gag-Pol requires a -1 frameshift during the process of translation. This occurs rarely, leading to a 20:1 ratio of Gag: Gag-Pol (107). This frame shift is mediated by two RNA structures, a 6-uracil sequence and a stemloop pseudoknot, ~200nt upstream of the Gag termination codon (41). Incompletely spliced mRNA encodes the Env, Vif, Vpr, and Vpu proteins. The substantial export of these unspliced and partially spliced mRNAs mark the establishment of late-stage gene expression.

1.1.2.8 Virion assembly and budding

The production of viral particles occurs during the late stage of viral gene expression. This process initiates with the assembly of virion components, protein and genomic RNA, at the cellular membrane. Particle assembly is dependent on the Gag and Gag-Pol polyproteins that target the plasma membrane and recruit additional viral and cellular factors to the assembling virions (107, 108). Once associated with the membrane, Gag undergoes a process of multimerization that drives the organization and assembly of the nascent virion (124).

The N-terminal MA domain of Gag is primarily responsible for the association of Gag and Gag-Pol with the plasma membrane. During translation, a myristic acid is attached to MA domain of the Gag polyprotein. At the plasma membrane, the MA domain binds to the

membrane lipid phosphatidyl inositol (4,5) biphosphate, exposing the N-terminal myristoyl group, called the myristoyl switch (242, 273, 274). This myristoyl group then inserts into the plasma membrane, anchoring the Gag or Gag-Pol molecule. These Gag proteins typically concentrate at detergent-resistant “lipid raft” regions of the membrane, with higher local concentrations of phosphatidyl serine, phosphatidyl ethanol, cholesterol, and sphingomyelin than the cellular plasma membrane in aggregate (44). These higher local concentrations are reflected in the membranes of the virions that bud from these sites (44).

Membrane targeting by MA localizes additional factors to the site of viral assembly. Uncleaved genomes bind the NC domain of Gag by means of a highly structured 150-nt RNA packing sequence, Ψ , located in the 5' UTR of the transcript (74). Recruitment of viral genomes to the assembly site also results in the recruitment of tRNA^{Lys3} which binds the viral genome and is required for reverse transcription following entry (180). MA also recruits the membrane-anchored viral envelope glycoprotein complex by means of an interaction with the cytoplasmic domain of Env (69, 345).

The high concentration of membrane-associated Gag and Gag-Pol results in multimerization of the molecules in a curved lattice. This multimerization, driven primarily by intermolecular association between CA and MA domains and dimerization of NC domains, creates a membrane-bound semi-spherical form that protrudes from the cell surface (55, 161). Beneath the membrane, the Gag molecules are ordered into a large lattice of CA hexamers (1, 327). The hexameric multimerization of the CA domain forces Gag into a radial arrangement with a nearly continuous shell of MA immediately adjacent to the membrane, an ordered shell of CA domains below the MA shell, and an NC domain shell beneath that. This “radial bead” lattice is the characteristic form of the immature capsid and is continuous over approximately

70% of a sphere (42, 114, 327). This capsid shell is discontinuous and absent at the site of viral membrane fission (327). This semi-sphere is composed of ~2500 monomers of Gag, and contains two copies of the viral RNA genome (55).

Release of the virion requires the involvement of components of the cellular ESCRT machinery which is responsible for the related process of membrane scission in the biogenesis of intra-endosomal vesicles (30, 56, 231, 251, 314). The p6 domain of Gag recruits ESCRT complex components, including tumor susceptibility gene 101 (TSG101) and ALG-2-interacting protein X (ALIX) to the site of fission (231, 297). These cellular cofactors, in turn, recruit additional members of the ESCRT complex mediating the separation of the membrane encasing the Gag semi-sphere, the nascent viral membrane, from the cell. An average of 7-14 Env trimer spikes are present within this cleaved membrane and are incorporated in the virion (62, 355).

In virus lacking Vpu, viral release is significantly inhibited by the cellular restriction factor tetherin. Tetherin anchors itself into both virions and the cellular membrane, preventing dissociation of virions even after membrane fission. Viral Vpu targets tetherin for degradation, substantially enhancing virion release (236, 237).

1.1.2.9 Viral maturation

Maturation of the virion occurs either concurrently or shortly following the release of the virion from the producer cell. Maturation is driven by the cleavage of Gag by the viral protease PR at 10 separate cut sites (258, 259). PR is active as a dimer; thus, the timing of maturation is likely dependent in part on the concentration of PR domains during the assembly of the immature capsid (185, 301).

Following cleavage by PR, the most prominent rearrangement occurs in the cleaved CA proteins. Approximately 1500 molecules of the cleaved CA proteins, about half of the CA molecules comprising the immature capsid, form a closed, conical form—the mature viral capsid (120, 124). The capsid is composed of ~250 CA hexamers and 12 pentamers that are required for a closed structure (24, 42, 123). Though the CA domains of Gag are arranged as hexamers in the immature capsid as well, the specific hexameric form is considerably different in the mature capsid and represents a significant rearrangement of the capsid lattice. Section 2.1.0 of this chapter includes a more detailed description of the mature capsid structure.

PR protease activity also has a significant impact on other elements of the virion. Following cleavage from P2, NC acts as an RNA chaperone, facilitating the dimerization of the two RNA genomes contained within the virion (72, 142, 162, 230, 267). SP1-NC cleavage also contributes to the activation of the viral spike proteins (233, 332). Following maturation, the virion is fully capable of binding and entering a new target cell, starting the viral lifecycle again.

1.2.0.0 Experimental investigation of capsid stability and uncoating

Following the release of the capsid into the target cell cytoplasm, the viral core engages in three critical processes: the release (or remodeling) of genome-associated CA, called “uncoating,” the reverse transcription of the viral genome, and the transport of that genome to the nuclear pore. Early literature on uncoating proposed that the process occurred at or near the plasma membrane almost immediately following the entry of the core into the cytosol (50, 167). Initial analysis of infected cells with transmission electron microscopy (TEM) found no evidence of intact capsids in the cytosol (134). Additionally, in purifications of viral RTC complexes, little to no CA protein was found to co-associate with viral genomes (167). This evidence taken

together gave rise to a theory that viral uncoating occurred immediately following entry, possibly initiated by the release of the concentrated CA protein following viral membrane fusion (47, 196).

In the past decade, two avenues of study have challenged the model of near-immediate uncoating. Firstly, refined experimental technique has indicated that CA protein may remain associated with viral RTCs far longer than initially thought (97, 222, 238). Secondly, studies of CA mutants and CA-associated host factors have demonstrated that premature uncoating of the capsid is detrimental to viral infectivity. Additionally, whereas it was once proposed that uncoating was necessary to initiate the process of reverse transcription, potentially by “releasing” viral genomes to encounter required host cell cofactors, more recent evidence indicates that the process of RT and uncoating temporally overlap and that the regulation of the processes may be interconnected (12). In this section, we will describe the specific assays that have pushed forward our understanding of viral capsid stability. In chapter 2, we present a novel capsid stability assay.

1.2.1.0 Study of the HIV viral core and *in vitro* capsid stability

The capsid element of the HIV-1 viral core is composed of ~1500 monomers of the CA protein arranged into a largely hexameric lattice (120, 123). Within the capsid shell, the core contains two copies of viral genome and multiple proteins (NC, IN, RT, Vpr). These viral proteins are responsible for the packaging of the genomes as well as supporting the reverse transcription and integration of the viral genome during infection. Multiple host factors have been determined to package with the core as well, including cyclophilin A and APOBEC3G (5, 106, 302, 348). Though only ~1500 CA monomers form the capsid, ~2500-5000 monomers of

CA are present in the virion (54, 55). Some of these free monomers are contained with the capsid, forming part of the viral core.

CA is a ~24-kDa protein composed of two domains: the N-terminal domain (NTD) and the C-terminal Domain (CTD) joined by a flexible linker region (26, 119, 122, 127, 229). The secondary structure of CA protein is primarily helical. The NTD contains seven alpha-helices; the CTD, four alpha helices (26, 256, 257). The core's ~250 hexameric rings are linked through NTD-NTD and NTD-CTD interactions of multiple CA monomers. The number of interaction sites between intra-ring CA binding partners is large, but helices 4, 7 (in the NTD), 8, and 11 (in the CTD) are the most important (64, 122, 127, 256, 257). Multiple hexamers are linked into a larger lattice by dimerization of the C-terminal region of the CTDs of two CA molecules present in different hexameric rings (51, 119, 257). The immature viral capsid formed by uncleaved HIV Gag prior to budding is largely spherical with a diameter of ~100 nm (42, 327). After cleavage of Gag by the viral protease, the mature core attains its canonical conical shape. Cores vary in size from 100-120 nm in length with a diameter at the cone's widest point of 50-60 nm (43, 65, 147, 295). The presence of 12 pentameric rings permits the lattice to form a closed structure (120). The asymmetric distribution of those pentamers generates the core's characteristic conical shape (120). The propensity of CA molecules to maintain this multimerized form is typically referred to as capsid stability; the disassociation of CA molecules from the other components of the viral core is referred to as uncoating.

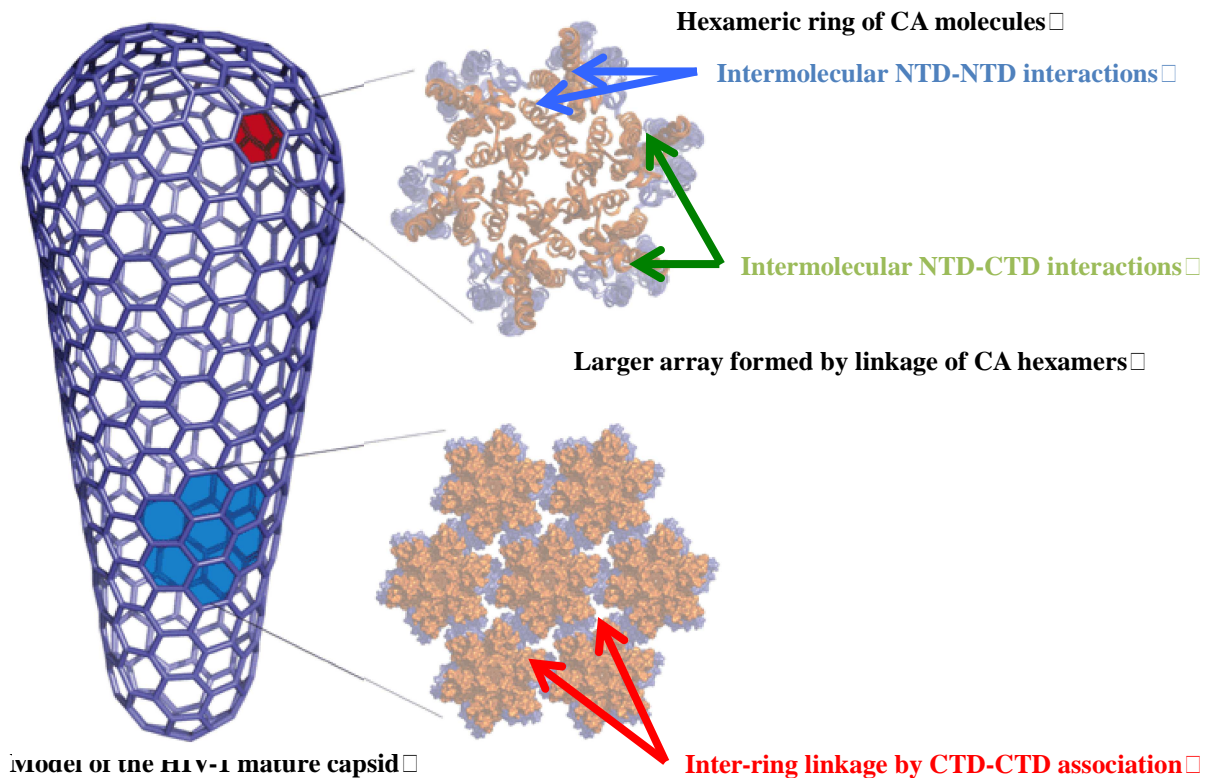


Figure 1.2 Structure of the mature HIV-1 capsid. The N-terminal and C-terminal domains of CA are colored orange and blue respectively. Original image by Stuart Borman (33) with X-ray data from Pornillos et al. (256)

Investigation of the retroviral capsid structure has been undertaken by multiple experimental approaches including x-ray crystallography and cryo-electron microscopy. One of the most valuable advances in the study of capsid structure was the development of a highly stable structural analog of the viral capsid, the CA-NC tube (53, 122). Purified CA-NC protein, when incubated at high concentration (300 μ M) and in the presence of high-ionic-strength buffer (0.5 M NaCl) and abundant nucleic acid fragments (6 μ M ssDNA 50-mers), assembles into large complexes of a hexameric lattice very similar in structure to the CA lattice of the mature capsid (120). These *in vitro* assembled structures are more stable than authentic purified capsids—likely due to the uncleaved NC domains that, with the ssDNA, enhance dimerization of the CA-NC proteins. Cryo-electron microscopy of these stable capsid structural analogs were crucial in

the creation of whole capsid structural models (122). Additionally, these CA-NC structures have proven valuable as capsid analogs in experimental analysis of CA-binding factors, including the assay described in chapter 2 (203).

1.2.1.1 Analysis of *gag* mutation affecting core stability

Alterations of CA affecting capsid uncoating can be broadly grouped into two categories: first, intrinsic stability alterations that decrease the multimerization potential of CA alone; and, second, cofactor association alterations that affect the interaction of capsids with cellular cofactors that contribute to the stabilization of the viral core. CA in the HIV-1 capsid makes intermolecular contacts at four regions: intra-hexameric NTD-NTD interface, an intra-hexameric NTD-CTD interface, an inter-hexameric CTD-CTD dimerization, and an inter-hexameric CTD-CTD trimeric interface (51, 119, 120, 124, 127). Inherent stability alterations of CA have been identified in all four of these regions (51, 102, 318, 336, 337, 346). CA protein with alterations at these sites have been shown to have decreased self-association outside of a cellular context as well as increased uncoating rates during infection. These intrinsic stability mutants have exhibited pre-integration defects as early as prior to completion of reverse transcription (88). Loss of intrinsic stability correlates well with a loss of infectivity (4, 102).

Alterations in CA may also decrease capsid stability by inhibiting the association of CA and core-stabilizing host factors or promoting association of CA and core-destabilizing restriction factors (202, 203). These alterations (*e.g.* those at the CypA binding site of CA) have demonstrated no significant defect relative to wild-type in *in vitro* capsid stability, but disassemble more rapidly *in vivo* in some cell types. Accelerated uncoating *in vivo* due to diminished CypA binding correlates well with decreased infectivity (202). Interestingly,

suppressor alterations for some intrinsic stability alterations have been reported to restore wild-type infectivity and (by one measure) *in vivo* uncoating dynamics, while retaining diminished core stability *in vitro* (337). This suggests that the virus may compensate for decreased intrinsic stability by making greater use of stabilizing host factors. While it is now clear that cellular factors also play a role in regulation of uncoating, initial investigations into uncoating focused on inherent capsid stability as a determinant of uncoating kinetics (102). The experimental approaches used to assess capsid structure and stability primarily examined the *in vitro* assembly and/or disassembly of CA protein.

1.2.1.2 *In vitro* assays of core stability / spin-through and cell-free fusion

Distinguishing inherent stability alterations from those that affect CA-cofactor binding requires an *in vitro* assay by which capsid stability can be measured in the absence of host factors. To accomplish this, viral cores are purified from virions by means of a spin-through protocol (102, 283). In this method, virions are first collected from producer cells and concentrated by ultracentrifugation. Concentrated virions are then loaded onto a two-part sucrose gradient/cushion. A 15% sucrose solution containing 1% solution of the detergent Triton X-100 is layered over a 30-70% sucrose gradient. The concentrated virus is then loaded over the 15% cushion and spun at 187,000 x *g* for 16 hours. During centrifugation and passage through the Triton X-100, the viral membrane is removed from the virion and the viral core enters the sucrose gradient. Delipidated cores are then isolated from the gradient. To assay core stability, the cores are diluted in neutral buffer and incubated over a range of time points (*e.g.* 0-120 minutes). Intact cores are then isolated from disassembled CA by pelleting of intact capsid by centrifugation at 125,000 x *g* for 20 minutes. Recovery of pelletable CA is subsequently

measured quantitatively by ELISA. The stability of individual cores is then assessed by the progressive loss of pelletable intact capsid across multiple time points.

A related protocol has been used to assess the effect of cellular factors on the uncoating of the retrovirus avian sarcoma and leucosis virus following cell-free fusion of the virion with isolated endosomes *in vitro* (235). In these experiments, virion membrane fusion is inhibited by the presence of ammonium chloride and virions within endosomes are specifically isolated for analysis. Following the removal of ammonium chloride *in vitro*, the viral membranes of endosome-contained virions fuse with the isolated endosomes, releasing delipidated viral cores in an *in vitro* cell-free environment. Experiments utilizing this technique indicate that ATP hydrolysis and unidentified cellular factors stimulate uncoating and viral DNA synthesis (235). However, this technique has not been widely adopted by researchers in the field and it is unclear if the results obtained are indicative of biologically relevant processes.

1.2.1.3 *In vitro* assays of core stability / purified CA assembly

Though the spin-through core purification method isolates viral cores from target-cell specific capsid-stabilizing factors, purified cores may still associate with stabilizing factors present in producer cells that are packaged within virions such as cyclophilin A. An alternate *in vitro* capsid stability assay uses viral CA purified from bacterial culture to avoid such complicating factors. In the CA assembly assay, recombinant CA protein is expressed in bacterial culture and purified by ion-exchange chromatography (192, 202). Purified CA protein incubated at a high concentration (44 μ M) in high-ionic-strength buffer (2.5 M NaCl) will assemble into large capsid-like complexes such as the CA-NC complexes described in section 2.1.0. The assembly of these complexes can be measured by an increase in 340 nm absorbance.

The maximum value and rate of increase of absorbance measurements in these assembly conditions can be used to assay the propensity of altered proteins to multimerize, a property directly associated with capsid stability.

1.2.2.0 Experimental approaches to the study of uncoating kinetics *in vivo*

Though *in vitro* assays of capsid stability have been important to the investigation of viral uncoating, there are significant associated drawbacks. *In vitro* assays provide little information regarding the timing and localization of capsid uncoating following entry. Additionally, barring a recent exception (284), *in vitro* assays have shown little usefulness in the evaluation of cellular cofactors' and restriction factors' contribution to uncoating. Investigation of these subjects required the development of *in vivo* uncoating assays.

The study of HIV viral uncoating *in vivo* has been challenging for reasons including, but not limited to, the instability of the viral core, the presence of unassembled CA protein within the virion, and an abundance of viral particles on non-productive pathways following cellular entry (12, 34, 59). To address these issues, researchers have developed several very distinct assays of viral capsid stability and uncoating. Unfortunately, despite the development of these assays, many fundamental elements of viral uncoating remain incompletely understood. The use of these multiple assays has resulted in very different estimates of the kinetics and location of viral uncoating (14, 152). Even within a given experimental framework (*e.g.* TEM analysis), findings have varied widely between investigators, potentially due the challenge of processing capsid-containing samples (12, 14, 134). This remains an extremely active area of research and the continued refinement of experimental protocols is critical to future progress.

1.2.2.1 Tracking CA-core co-association during infection / fractionation of cytoplasm

The earliest investigations of capsid uncoating involved tracking the association of CA protein with reverse-transcription-competent complexes in the cytoplasm of infected cells. To isolate these RTC/PICs and identify the associated viral proteins, ³H-arginine-labeled virions were added to target cells (50). Four to five hours later, target cells were lysed and cytoplasmic extract collected. These cytoplasmic extracts were then fractionated by Sephracyl S400 chromatography and sucrose gradients of 15-30%. These fractionation steps allowed partial separation of the viral RTC/PICs from unassociated factors also present in the cytoplasm. The RTC/PIC-containing fractions were identified by Southern Blot for reverse transcription products. These RTC/PIC-containing fractions were then assayed for the presence of ³H-labelled viral proteins. In these experiments, the viral CA proteins were present in only low concentration in viral DNA-enriched fractions. Additionally, CA was most abundant in fractions that contained no viral DNA. These findings suggested that reverse transcription occurred following uncoating and that, therefore, uncoating must be completed early in the viral life cycle (50, 95, 167, 226).

Subsequent research indicated that CA plays a critical role in processes that take place later in infection than would be possible in the model of very early-stage uncoating suggested by this data (11, 96, 186, 334). It is likely that this experimental protocol poorly measures the co-association of CA with infectious RTC/PICs, potentially due to the instability of the viral core. The process of fractionation may be sufficient to induce capsid disassembly and CA disassociation from the viral genome and reverse transcription products. Additionally, interpretation of this experiment was likely made more challenging by the presence of CA in non-productive pathways. Viral capsid that had been endocytosed or otherwise removed from

the productive infection (estimated to be >85% of intracellular CA (216, 222, 303)) pathway likely formed the large CA-containing complexes that lacked reverse transcription products.

1.2.2.2 Tracking CA-core co-association during infection / *in situ* fluorescent staining

In order to compensate for the instability of the viral capsid, more recently developed protocols of this type avoid the fractionation of cytoplasmic components and track the co-association of the RTC core and CA *in situ* (52, 152, 335).

One method of CA-core *in situ* tracking involves the fluorescent labeling of viral components and the visualization of those components by microscopy during infection. In this protocol, the viral membrane is labeled with S15-mCherry and the viral core with GFP-Vpr (222). Following infection, target cells are fixed at various time points and CA visualized by staining with fluorescently labeled anti-CA antibody. The co-association of CA and the viral core is determined by comparison of anti-CA antibody fluorescence and Vpr-GFP fluorescence at multiple time points. This method eliminates some of the challenges resulting from the presence of non-productive capsids, as endocytosed capsids are identifiable by the S15-mCherry labeled membranes that surround them. In contrast to the findings of RTC isolation protocols described above, *in situ* experiments have demonstrated co-association of CA with RTCs to be common and persistent. In one study, greater than 62% of fusion-released cores were associated with CA 1 hour post-infection with approximately 44% and 32% still CA-associated at 2 and 4 hr post-infection respectively (152). Additionally, while earlier models of uncoating proposed that uncoating occurred primarily at the plasma membrane shortly after entry, tagged core analysis indicated the presence of CA-associated RTCs on microtubules, suggesting that uncoating occurs during or after the transport of the viral cores to the perinuclear space (152).

1.2.2.3 Tracking intact capsid during infection/ electron microscopy

Researchers have also sought to follow post-entry core uncoating by tracking the core's characteristic large CA protein complex itself. These methods have relied upon either the detection of conical core by microscopy or isolation of large CA-containing complexes by density fractionation. The conical CA-containing viral core is readily apparent by transmission electron microscopy of HIV-1 virions. However, visualization of the core following entry into the cytoplasm has proven far more difficult, likely due to the decreased abundance of cores in these samples and the transience of assembled core following target cell entry (6, 14, 134, 270).

To enhance detection of intracellular cores, researchers employ experimental methods to increase local concentration of intact capsids. One group has reported the ability to identify conical cores post-infection by HIV-1 virus lacking the central DNA flap (14). The absence of this DNA flap, a three-stranded DNA structure present in the completely reverse-transcribed genomes, has been reported to inhibit import of the viral cDNA into the nucleus and restrict infection (13, 14). It was proposed that the absence of this flap halted infection pre-uncoating, increasing the abundance of intact viral capsids for visualization. These more abundant viral capsids were then observed as intact cores localized on the cytoplasmic face of the nuclear membrane in proximity to the nuclear pore complex (14). In this same study, additional analysis of CA at the nuclear membrane by confocal immunofluorescent microscopy and scanning electron microscopy indicated that during HIV-1 infection, CA accumulation in the proximity of the nucleus peaked at 24 hours, diminishing substantially by 48 hours. This contrasts significantly with the models of comparatively rapid uncoating suggested by analysis of CA-core co-association. Data collected by this method has led to a model of capsid uncoating in which

cores remain intact throughout transportation to the nucleus, shedding capsid only after completion of reverse transcription, several hours after association with the NPC (12, 14).

The visualization of the intact conical core provides a very direct method of tracking the progress of the viral cores following entry; however, there are several significant associated challenges. It remains unclear that the slowly disassembling capsids visualized by these methods are the source of infectious genomes and not aberrantly stable complexes. The assay is technically difficult and low-throughput. Consequently, the assay has not experienced wide adoption in the field. Additionally, the relative scarcity of observable intact particles in cells has made the study of virus without mutant DNA flap, or other uncoating defect, challenging. Given these challenges, it has been difficult to determine the accuracy of the “at the NPC” models of capsid uncoating developed from the data generated by these protocols (12, 14).

1.2.2.4 Tracking intact capsid during infection/ fate-of-capsid

An alternate method of tracking the CA component of viral cores is achieved by the separation of multimerized CA protein by sucrose density fractionation. This assay, called fate-of-capsid, utilizes the greater density of viral cores relative to unassembled CA protein to track the disassembly of intact capsid into its components during uncoating. To measure the rate of uncoating, cytoplasmic extracts are collected at multiple time points from parallel infections (299). Cytoplasmic extract from each time point is then centrifuged at $125,000 \times g$ over a 50% sucrose cushion. During the spin, high-density assembled cores (~ 1.25 mg/ml) are separated from non-productive endocytosed capsids in lysosomes (approximate density 1.2 mg/ml) as well as disassembled CA protein, which lacks the sedimentation rate necessary to pass through the sucrose cushion. High-density core-associated CA complexes are compared across this time

course by western blot of pellet fractions following ultracentrifugation. Similar to the microscopy studies of nucleus-associated CA, described above, these assays showed the most significant loss of CA occurred from time-points between 24 and 48 hours post-viral incubation (299). However, as fusion in these assays is asynchronous, these time points may overstate the persistence of the viral core post-entry. Fate-of-capsid assays that account for asynchronous fusion have estimated the half-life of EIAV core disassembly to be 8-12 hours (Bollman, unpublished). Traditional fate-of-capsid assays follow only CA protein, but a recent similar protocol permits probing of these high-density CA complexes for the presence of additional components of the viral core (188).

The fate-of-capsid assay is particularly useful in the parallel comparison of uncoating rates by dissimilar viruses or in dissimilar cellular contexts. The assay was initially developed to study the effect of the viral restriction factor TRIM5 α . That assay demonstrated that TRIM5 α induced premature uncoating of HIV-1 and multiple other retroviruses (82, 253, 299, 337). Since its development, the assay has been utilized to evaluate the effects of both *gag* mutations and cellular factors thought to promote capsid stabilization or uncoating (113, 157, 202, 288, 337).

1.2.2.5 Measuring uncoating by capsid sensitivity/ CsA addition and CsA washout

The progressive disassembly of cores has also been investigated by analyzing the sensitivity of infectious particles to host cofactors and restriction factors. Two separate methods of core stability tracking have been developed. Somewhat confusingly, the two rely on opposite effects of the drug cyclosporin A (CsA) on HIV-1 infection in specific cellular contexts. The first method, the CsA addition protocol, depends upon the ability of CsA to inhibit HIV-1

infection in specific cell types (11, 335). CsA reduces viral infectivity by inhibiting binding of CA to cyclophilin A (106, 208), a critical cofactor of infection in some cell types (38, 105, 106, 170, 302, 319). For this reason, sensitivity to CsA addition is used in this assay as a measure of CA association with the infectious RTC. The stated assumption of this assay is that RTCs that are no longer associated with CA are insensitive to inhibition by CsA. In the assay, multiple identical cells cultures are infected in parallel with luciferase or GFP-tagged HIV-1 reporter virus. Individual preps are then treated with CsA beginning at multiple time points following infection. Addition of CsA at time points soon after infection led to reduced infectivity with a $t_{Stab50\%}$ (the CsA addition time point corresponding to 50% of the infectivity of the no CsA control) ranging from 6-24 hours depending on cell type (11, 335). By measuring infectivity, this assay avoids one of the persistent challenges of experimental study of capsid uncoating—only virions on a productive pathway contribute to the output data. However, the assay does depend on CsA sensitivity as a true measure of intact capsid; it is currently unclear if this is the case or if CsA sensitivity diminishes in advance of the completion of uncoating.

A somewhat similar assay, the CsA washout assay, depends on the ability of CsA to inhibit the restriction of HIV-1 infection by the host restriction factor TRIM-Cyp (252). Like that of cyclophilin A, the binding of TRIM-Cyp to HIV CA is inhibited by CsA (101, 239, 277). TRIM-Cyp binds to, and thus inhibits infection by, only those cores that still contain CA protein. In the protocol, parallel preparations of TRIM-Cyp expressing cells are infected with reporter virus in the presence of CsA. At multiple time points following infection, media from individual preparations is replaced with media lacking CsA. Removal of CsA from infected cells over a time course indicated that over 50% of infectious particles were TRIM-Cyp insensitive 1 hour post-infection, with nearly all particles TRIM-Cyp insensitive by 4 hours post-infection (152).

This measure of uncoating kinetics is in line with that modeled by *in situ* measurement of CA-RTC co-association (section 2.2.1.2), far faster than that suggested by measurement of large CA-containing complexes or CsA sensitivity (section 2.2.2/2.2.3). This assay carries a similar caveat to the CsA addition protocol. Though it is clear that TRIM-Cyp cannot restrict a viral core which has lost all CA protein (252), it remains undetermined if a core may lose TRIM-Cyp sensitivity prior to the completion of uncoating.

1.2.3.0 Study of cellular capsid-stabilizing and capsid-destabilizing factors

The development of the HIV-1 capsid stability and uncoating assays discussed above has been crucial to the investigation of cellular factors that contribute to the regulation process. In the following chapters, we present a novel *in vitro* capsid stability assay that we used to characterize a putative capsid-stabilizing cofactor, PDZ domain-containing protein 8 (PDZD8). Here, we briefly introduce the cellular factors thought to promote or inhibit HIV-1 core uncoating, including PDZD8.

1.2.3.1 TRIM5 α and TRIM-Cyp

One of the most significant catalysts in the expansion of knowledge regarding retroviral capsid stability over the past decade has been the characterization of the cellular restriction factor tripartite motif 5 α (TRIM5 α). The identification of TRIM5 α as a determinant of target cell susceptibility to specific retroviruses and the finding that TRIM5 α inhibits infectivity by enhancing viral capsid uncoating were important to establishing the theory that proper regulation of capsid uncoating is required post-entry (298, 299).

Prior to the characterization of TRIM5 α as a restriction factor, a post-fusion, pre-integration block of multiple retroviruses was described in humans (called Ref1) and other non-human primates (Lv1) (27–29, 70, 232, 306). Lv1 was noted to be a potent inhibitor of HIV-1 infection in Old World monkeys. In 2004, a screen of Old World monkey cDNA identified TRIM5 α as the Lv1 restriction factor (298); human TRIM5 α was identified as Rev1 shortly thereafter (254). Subsequent research, including the development of the aforementioned fate-of-capsid assay, provided evidence that TRIM5 α restricts infectivity by inducing the premature disassembly of targeted retroviral capsid (299)

TRIM5 α is a cytoplasmic member of the TRIM family of proteins and is ~500 aa in length (268). There are over 100 TRIM proteins identified and several, like TRIM5 α , have been shown to play a role in the processes of innate immunity (80, 173, 240, 247). TRIM proteins are defined by the presence of three characteristic motifs—the N-terminal RING domain, one or more B-box motifs, and a coiled-coil motif (225). In addition to these three motifs, TRIM5 α also features a C-terminal SPRY domain that is required for retroviral restriction (73, 282, 299).

To restrict retroviral infection, TRIM5 α must bind the CA protein of the assembled retroviral capsid. The SPRY domain is primarily responsible for this binding (158). Ability to evade recognition by TRIM5 α SPRY domains is a significant determinant of TRIM5 α insensitivity (and consequent infectivity) of retroviruses within a specific host organism. For this reason, though many primate TRIM5 α proteins demonstrate an ability to recognize multiple retroviruses, recognition of retroviruses endemic to that species is typically low (140, 175, 254). Human TRIM5 α binds and restricts HIV-1 poorly (298). However, a single amino acid mutation within the SPRY domain is sufficient to induce substantial capsid binding and restriction (203). The CA-binding function of SPRY can be replaced by other CA-binding motifs as well,

preserving restriction (195, 279). In owl monkeys, an insertion event resulted in a TRIM5-cyclophilin (TRIM-Cyp) fusion protein in which the HIV-1 CA-binding protein cyclophilin A has replaced the SPRY domain. TRIM-Cyp restricts infection by cyclophilin-binding lentiviruses (261, 277, 316)

The specific mechanism by which TRIM5 α induces premature uncoating is unknown, but the RING, B-Box, and coiled-coil motifs all appear necessary for efficient restriction. Evidence suggests that restriction is dependent on the multimerization of TRIM5 α and that all three domains as well as the region linking the coiled-coil to the SPRY domain contribute to this multimerization (157, 200, 201). Intermolecular association of the TRIM5 α coiled-coil domains mediates dimerization (158, 166), while both RING and B-Box domains appear to be required for the formation of higher-order multimers (83, 200, 201). Electron microscopy of a TRIM5 α -TRIM21 fusion construct (TRIM5 α , with the substitution of the TRIM21 RING-domain) showed evidence of large, regular hexagonally-ordered complexes (121). The formation of a similar large multimeric form by endogenous TRIM5 α has been proposed to both enhance recognition of the multimeric CA lattice of capsid and contribute directly to the premature uncoating of the capsid by an as yet unidentified mechanism (121).

1.2.3.2 CPFS6/TNPO3

Cleavage and polyadenylation specificity factor 6 (CPSF6)—a 551 aa component of the nuclear RNA cleavage machinery—is predominantly localized within the cell nucleus, likely due to the action of cellular nuclear transport factor TNPO3 (171). However, when CPSF6 is localized to the cytoplasm by mutation or inhibition of TNPO3 function, CPSF6 is capable of inducing potent restriction of HIV-1 infectivity by hyperstabilizing the viral capsid (78, 194).

CPSF6 was first identified as a potential HIV-1-interacting factor in a cDNA expression screen (194). A fragment of CPSF6 with a significant C-terminal truncation (CPSF6-358) was found to strongly restrict VSV-G-pseudotyped HIV-1. The truncated region of CPSF6-358 includes the protein's nuclear localization sequence. Consequently, the truncated form, unlike full-length CPSF6, is localized to the cytoplasm. Truncated CPSF6 and other CPSF6 mutants that are cytoplasmically localized bind HIV CA protein and inhibit completion of reverse transcription or nuclear import of the viral PIC (149, 194). This restriction is dependent on the binding of CPSF6 residues 314-322 within a channel formed by helices 3,4, and 5 of HIV CA protein (260). Binding of cytoplasmic CPSF6 to the viral capsid slows the uncoating of the capsid, as determined by the fate-of-capsid assay (78, 113). Additionally, the interaction of HIV-1 and CPSF6 has been recently reported to inhibit recognition of the virus by cellular sensors that stimulate innate immune responses (265).

The study of CPSF6 and HIV-1 has dovetailed with that of TNPO3. TNPO3, a nuclear import receptor for serine/arginine rich proteins (171, 190), was first identified as an HIV-1-interacting factor by an siRNA screen; knockdown of TNPO3 significantly decreases infection of targeted cells by multiple retroviruses (40, 183). Though TNPO3 was initially identified as an IN-interacting factor (64), subsequent work demonstrated that TNPO3 knockdown sensitivity is dependent on interaction with the viral capsid (77, 186, 352). Sensitivity of HIV-1 to TNPO3 appears to be dependent on CPSF6; knockdown of both factors simultaneously restores the HIV-1 infectivity lost by knockdown of TNPO3 alone (78, 113). One model proposes that in the absence of the import factor TNPO3, full-length CPSF6 localizes within the cytoplasm, restricting viral infectivity by hyperstabilizing the viral core. In support of this model, TNPO3

knockdown has been reported to increase levels of cytoplasmic CPSF6 (78); however, this finding has been disputed (113).

In addition to its potential role in regulating capsid stability by maintaining CPSF6 nuclear localization, TNPO3 has been hypothesized to play a direct role in stimulating capsid uncoating (284). In an *in vitro* assay of capsid stability, purified TNPO3 alone, in the absence of CPSF6, enhanced capsid uncoating (284). Purified TNPO3 also enhanced the potency of the capsid-destabilizing small molecule PF74 and countered the stabilizing effect of the cellular cofactor cyclophilin A (284). Additionally, knockdown of TNPO3 appears to enhance accumulation of viral CA protein within the nucleus (352). Given this evidence, it has been hypothesized that TNPO3 acts as a post-entry uncoating factor, acting to remove residual CA protein from the PIC in advance of integration (284).

1.2.3.3 Cyclophilin A

Prior to the identification of PDZD8 as an HIV-1-interacting factor, the only known cofactor of HIV-1 infection that stabilizes the viral capsid was cyclophilin A (202). CypA was first identified as an HIV-1-interacting factor following the finding that the drug cyclosporine A inhibited HIV-1 infection (319). Subsequent work identified the CsA-sensitive viral cofactor CypA as the cellular protein responsible for this drug response (208).

CypA is peptidyl-prolyl isomerase that catalyzes the cis-trans isomerization of proline imidic peptide bonds (131, 163, 174). Cyclophilin A was first discovered to be a Gag-binding protein by a yeast two-hybrid screen (208). Later work identified an exposed loop of the CA protein (aa 85-93) as the specific site of CypA binding (118, 121, 315, 350). The interaction of Gag CA domain and CypA leads to the specific incorporation of CypA into the viral capsid at a

stoichiometry of approximately 1 CypA molecule: 10 Gag molecules (106, 302). However, incorporation of producer cell CypA does not appear to be necessary for productive infection (293).

HIV-1 sensitivity to CsA is due to the action of target cell CypA in the early, pre-integration steps of infection before or during reverse transcription (139, 293). CypA enhancement of infection is dependent upon CA-CypA binding. Initially it was hypothesized that proline cis-trans isomerase activity of CypA stimulated the uncoating of the viral capsid (3, 85). The active site of CypA binds a compatible peptide proline bond (G89-P90 of CA) though it is not known to what degree the binding induces cis-trans isomerization (35, 118, 343).

The development of the fate-of-capsid assay allowed expanded analysis of the role of CypA on CA stability (202). This investigation revealed that, in cell lines in which HIV-1 is CsA-sensitive, CypA enhances infectivity by stabilizing the viral core (202). The specific mechanism by which CypA stabilizes the viral core is not well understood; nor is it known whether the enzymatic properties of CypA are required for this effect.

CypA enhancement of HIV-1 infection is not found in all permissive cell types. CsA inhibition of CypA-CA binding reduces infectivity in Jurkat immortalized T lymphocytes, macrophages, and primary blood lymphocytes (39, 139, 293). However, CsA treatment exerts minimal effect on pre-integration infectivity of HIV-1 in HeLa cells (38). Additionally, some *gag* mutant HIV-1 viruses are CsA dependent for infection in specific cell types (38, 336). CypA binding to these viruses appears to stimulate premature uncoating, potentially by presenting steric impediment to the capsid lattice (133, 202, 324). It has been proposed that CypA may then act as both stabilizing factor and destabilizing factor. Difference of CypA

concentrations among cell types as well as affinity of CA proteins for CypA may mediate this biphasic response (202, 340, 341).

CypA-CA interaction is well conserved, though not universal, among lentiviral lineages and apparently quite ancient. Study of archaic retroviral capsids has indicated that CypA binding by lentiviruses was likely present more than 12 million years ago (130). CypA-CA binding is widely conserved among lentiviruses; however, notably, SIVmac CA does not bind CypA (106, 302).

1.2.3.4.0 PDZD8 introduction

In subsequent chapters of this dissertation, we will present the first evidence that human PDZD domain-containing protein 8 (huPDZD8) slows the process of HIV-1 uncoating *in vivo* and *in vitro*. The PDZD8 protein is ~129-kDA in size (1154aa) and is encoded by the human *PDZD8* gene located on chromosome 10. The PDZD8 gene is well conserved among mammals. Identified mammalian PDZD8 orthologs include those in chimpanzee (99.5% identical to human PDZD8 by amino acid sequence comparison), dog (90.7% identical) and mouse (87.4% identical). The gene is relatively well conserved among animals, with orthologs also identified in birds (chicken *PDZD8*, 65.9% identical), fish (zebrafish *PDZD8*, 60.3% identical), insects (mosquito *AgaP_AGAP002576*, 34.3% identical), and roundworms (*C. elegans* C53B4.4, 28.5% identical). Sequence analysis of PDZD8 identified three conserved domains: a PDZ domain, a Protein Kinase C-like zinc-finger (PKC1) domain, and a putative coiled-coil motif.

Currently, there is almost no information about the function of PDZD8 *in vivo*, but some evidence suggests the protein may interact with factors associated with cytoskeletal networks (144). Multiple members of the PDZ-domain-containing family of proteins have been shown to

interact with members of the ERM protein family, three highly-related paralogs (ezrin, radixin, and moesin) that link the plasma membrane to actin filaments (98, 310). These interactions, mediated by the PDZ domain, often form multi-molecular signaling complexes (138). Recently, huPDZD8 itself was reported to co-precipitate with the protein ERM-family member moesin (144). In addition to crosslinking actin filaments and the plasma membrane, moesin has been shown to negatively regulate a stabilized type of microtubule filaments, detyrosinated microtubules (Glu MT) (234). PDZD8 has been reported to induce a similar effect. Overexpression of PDZD8 decreased the abundance of stabilized Glu MT filaments, while siRNA knockdown of PDZD8 increased levels of Glu MT (144).

PDZD8 has also been tenuously linked to a hereditary disease in cows (219). A specific forelimb girdle muscular anomaly (FMA) in a population of Japanese cattle was tightly linked to 3.4 Mb locus containing the bovine *PDZD8* gene (219). The anomaly is characterized by growth retardation, tremors, and hypoplasia of muscles localized in the forelimb girdle muscles, with the musculature of other regions unaffected. Linkage analysis identified the *PDZD8*-containing region of chromosome 26 (a region with conserved synteny with chromosome 10 in humans) as the site of the heritable defect (219). However, the identified region contains multiple genes. PDZD8 was highlighted as a potential site of mutation due to the role of other PDZ-domain-containing proteins in cytoskeletal regulation, but no specific defect in PDZD8 expression or regulation was identified. Symptomatically similar diseases, limb girdle muscular dystrophies (LGMD), have been described in humans but linkage analysis has implicated regions on human chromosomes with no conserved synteny to the identified cow chromosome 26 site and no evident relationship with PDZD8 (18, 23, 91, 135, 153, 204, 227, 250).

1.2.3.4.1 PDZD8 and HIV-1

PDZD8 was first identified as a potential HIV-1-interacting factor by a yeast two-hybrid screen(143). A fragment of PDZD8 containing the protein's predicted coiled-coil motif (aa 932-1119) bound a bait molecule containing a fragment of HIV Gag (aa 59-250). This region of Gag is composed of both the C-terminus of MA and N-terminus of the CA protein (143). In subsequent chapters, we will present evidence that PDZD8 is binding the HIV CA region. Full-length HIV-1 Gag co-immunoprecipitates with full-length PDZD8, but this binding is lost with a C-terminal truncation of PDZD8 that removes the coiled-coil motif (143). It has been reported that PDZD8 also has a functional role in enhancing HIV-1 infection. Overexpression of PDZD8 enhanced VSV-G pseudotyped HIV-1 infection in both human brain microglia CHME3 and kidney 239A cell lines (143). Similarly, knockdown of PDZD8 expression by siRNA reduced HIV-1 infectivity in both cell lines. Additionally, it was demonstrated that the PDZD8-overexpression-driven infection enhancement effect occurred soon after entry with a corresponding increase in early RT products. The effect appears dependent on HIV CA binding: both infection and eRT product enhancement were lost with truncation of the PDZD8 C-terminal region containing the coiled-coil. PDZD8 overexpression also enhanced infection by VSV-G pseudotyped Moloney murine leukemia virus, (Mo-MLV) and simian immunodeficiency virus (SIVmac) (143). Interestingly, although siRNA knockdown of PDZD8 inhibits HIV-1 infection, PDZD8 was not identified as an HIV-1 cofactor by any of several previous high-throughput siRNA screens for HIV-1 cofactors (40, 129, 183, 351)

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1.2.3.4.2 Moesin, HIV, and PDZD8

In addition to HIV Gag, PDZD8 has been shown to interact with the host factor moesin, which is a pleiotropic regulator of HIV-1 infection (99, 144, 187, 287). Multiple interactions between HIV viral proteins and moesin have been described, but the overall role of moesin in infection is incompletely understood (22, 234). In T-cells, HIV infection upregulates expression of moesin and moesin has been reported to be specifically incorporated into virions (246). Knockdown of moesin by siRNA has been reported to enhance infectivity of HIV-1 and VSV-G pseudotyped HIV-1 in multiple cell lines (234). However, siRNA knockdown has also been reported to inhibit infection by some, but not all, X4-tropic strains (22). It has been proposed that these opposite effects of moesin may be due the action of moesin in multiple steps of infection. Moesin-mediated actin polymerization appears to enhance viral binding or entry, but moesin evidently also inhibits some post-entry steps in the viral lifecycle (187). The mechanism by which moesin inhibits HIV infection post-entry is not well understood. As with PDZD8, siRNA silencing of moesin increases the stability of stable Glu MT filaments (234). Surprisingly then, the effect of moesin overexpression on HIV-1 infection is opposite that of PDZD8; moesin overexpression decreases both HIV-1 and Mo-MLV infectivity (187). The moesin-mediated block, like the PDZD8-mediated enhancement, occurs after virus entry but precedes the production of early RT products. Further investigation of the functional relationship between PDZD8 and moesin may help to clarify the mechanisms by which PDZD8 enhances retroviral infectivity.

1.2.3.4.3 Domains of PDZD8

These are currently no molecular structures available for human PDZD8 or its orthologs. However, sequence analysis has identified several predicted domains/motifs that informed the experiments described in subsequent chapters of this dissertation. Analysis of human PDZD8 identifies three likely conserved domains: a PDZ protein-binding domain (aa 366-449), a protein kinase C-like zinc finger (aa 840-890), and a putative coiled-coil motif (aa 1028-1063). Preliminary investigation of PDZD8 truncation mutants (see chapters 3) indicate that the coiled-coil motif is critically important to the factor's role in slowing the rate of HIV capsid uncoating.

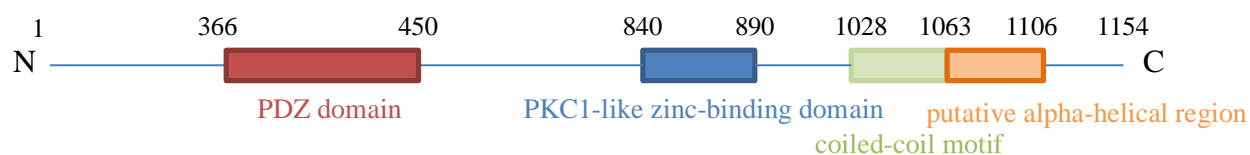


Figure 1.3 Predicted structural elements of PDZD8.

Canonical PDZ (PSD95-Discs Large Zonula Occludens) domains are 80-90 residues in length and characterized by the presence of six beta strands and two alpha helices (84, 90, 209). The PDZ domain frequently acts as a site of protein-protein binding, typically selectively binding the C-terminus region of binding partners (184, 281, 294), though internal peptide sequences and phosphatidylinositol membrane lipids have also been identified as PDZ-binding partners (115, 321, 356). PDZ-domains are common: 267 have been identified in the human genome distributed over 150 proteins (209). Protein interactions mediated by PDZ-protein binding are critical to many cellular systems including cell polarity and protein trafficking (287).

No binding partner of PDZD8's PDZ domain has been experimentally confirmed, but the binding characteristics of PDZ domains in general are well studied and models have been

developed to predict potential binding partners by primary sequence analysis (305). The globular PDZ domain binds the target short linear motifs (SLiMs) using a groove between the β 2 strand and α 2 helix, with the binding SLiM forming an antiparallel β sheet with the β 2 strand (84, 209). In PDZD8, this groove is predicted to be formed by residues 376S, 377V, 378G, 379L, and 381L (β 2) and 430V, 431L, 434I, and 435K (α 2) (176). Based on this sequence, PDZD8 appears to most closely resemble Class II PDZ-domains, which have a canonical binding motif of X Φ X Φ -COOH (193). One newer model predicts the more specific target sequence of [S/R][hydrophobic][W/R][L/I]-COOH (176). In chapter 3, we present evidence that while deletion of the PDZ domain-containing N-terminal region of PDZD8 abrogates HIV-1 core stabilizing function, the perturbation of the putative SLiM-binding site by mutation of β 2 and α 2 residues does not.

Protein kinase C-conserved region 1 (PKC1 or C1) domains are ~50 aa in length and characterized by two cysteine-rich zinc binding sites and a diacylglycerol (DAG) binding site formed between two surface loops. In PDZD8, the putative DAG-binding site is defined by residues 848-852 and 861-865. In some well studied C1 domain-containing family members, binding of the secondary messenger DAG or analogous phorbol esters (P/E) induce localization of those proteins at the cell membrane by serving as hydrophobic anchors (264, 285). DAG or P/E binding appears to fill the protein's hydrophilic P/E-binding groove, dramatically increasing the hydrophobicity of the C1 domain and further promoting membrane association (90). In these proteins, binding of cations is required to support DAG or P/E binding (243). Human PDZD8 has a putative zinc-binding motif comprised of residues H841, C854, C857, C869, C872, and H877. The intracellular localization of PDZD8 is still largely unstudied, and it is not known if DAG or P/E binding stimulates membrane association of this protein. Our research indicates

that the putative P/E binding domain is not crucial to stabilization of the HIV core. Deletion of the PKC1 domain from PDZD8 does not eliminate the ability of the protein to enhance HIV-1 infectivity or *in vitro* stability (chapter 3).

The putative coiled-coil domain of PDZD8 is located from aa 1028-1063, though additional alpha-helical tendency is predicted from residues 1063-1106. Coiled-coil domains frequently self-associate in dimers, trimers, or tetramers (218). Primary sequence analysis predicts that this region will have a propensity to self-associate as dimers (210). In my own research with cross-linked purified peptide fragments of this coiled-coil region, the fragments do form dimers and higher-order multimers (chapter 4). However, it remains unclear if full-length PDZD8 readily multimerizes.

The C-terminal region of PDZD8 including the putative coiled-coil motif has been implicated in the interaction of the protein with both HIV CA and moesin, PDZD8's only identified binding partners (144). Fragments of the C-terminus containing the coiled-coil motif were identified as binding partners of both HIV Gag and moesin bait constructs by yeast-2 hybrid screens. Truncation fragments lacking the putative coiled-coil motif, do not co-IP with HIV Gag or enhance HIV-1 infectivity (143). In chapter 3, we present evidence that indicates that the coiled-coil motif is necessary and sufficient for the binding of PDZD8 to multimeric arrays of HIV-1 CA protein. This region is also necessary (but not sufficient) for stabilization of HIV capsid analogs *in vitro* and HIV cores *in vivo*.

1.3.0 Current investigations

In chapter 2, we describe a novel *in vitro* assay measuring the capsid-stabilizing ability of cellular lysates. Previously developed *in vitro* assays of retroviral core stability have shown little

ability to capture the stabilizing and destabilizing effects of cellular cofactors of viral uncoating. This has been hypothesized to be due in part to the limited stability of purified viral cores *in vitro* (102, 283). The assay described in chapter 2 makes use of *in vitro* assembled HIV-1 CA-NC protein, which forms multimers with capsid-like structural properties, as a capsid analog. The increased stability of CA-NC complexes, relative to delipidated cores, allowed us to develop an *in vitro* assay more compatible with investigation of cellular cofactor contribution than previously described assays.

The use of the CA-NC stability assay indicated the potential existence of a previously uncharacterized HIV-1 capsid stabilization activity in human cell lysates. Incubation of CA-NC complexes in cytoplasmic extracts slows the progressive disassembly of large, quickly sedimenting CA-NC tubes into smaller complexes. We demonstrated that cytosolic lysates from selected human, dog, mouse, and chicken cell lines, but not *E. coli* bacterial lysates, are capable of stabilizing CA-NC tubes *in vitro*. To identify the factor(s) responsible, we performed these stabilization assays with lysate fractionated to isolate specific cytoplasmic components. Our research indicated that the factor(s) responsible are likely one or more proteins >100-kDa in size.

In chapter 3, we present evidence that PDZD8 is an important stabilizing component in cellular lysate. PDZD8 has been previously described as a positive HIV-1 cofactor of unknown function (143). To evaluate the role of PDZD8 in capsid stabilization, we treated HeLa cells with PDZD8-targeting siRNA. Lysate from cells with this targeted siRNA stabilized CA-NC complexes more poorly than lysate treated with negative control siRNA. Additionally, add-back transfection with vectors expressing siRNA-resistant PDZD8 clones restored CA-NC stabilization.

We also present evidence that the *in vitro* stabilization of CA-NC by PDZD8 correlates well with *in vivo* stabilization of retroviral cores. siRNA knockdown of PDZD8 reduces HIV-1 infectivity and HIV-1 core stability as measured by the fate-of-capsid assay. We found that PDZD8 knockdown reduces infectivity of multiple other retroviruses: simian immunodeficiency virus, feline immunodeficiency virus, and Moloney murine leukemia virus but not Rous sarcoma virus.

Finally, we include data from an investigation into the contribution of specific regions of the PDZD8 protein to the observed retroviral stabilization effect. Deletion of the PDZ-domain-containing N-terminal region and the C-terminal coiled-coil motif both abrogate PDZD8's stabilization activity. The add-back expression of PDZD8 constructs lacking these regions can restore neither the poor *in vitro* stabilization nor the infectivity defect of cells treated with PDZD8-targeting siRNA. The coiled-coil region in particular is required for binding of PDZD8 to the HIV-1 CA protein.

In sum, this research provides the first evidence that PDZD8 acts as CA-binding capsid-stabilizing cofactor of HIV-1 infection. Prior to this finding, only CypA had been identified as such a factor. Though the mechanism by which PDZD8 stabilizes retroviral capsids remains unknown, the development of the *in vitro* CA-NC stabilization assay offers a promising tool for future investigation. Previous *in vitro* assays have shown little ability to replicate *in vivo* cofactor-capsid stabilization effects. With this assay, however, we have demonstrated a strong correlation between PDZD8 construct stabilization of CA-NC *in vitro* and capsids *in vivo*. The development of this *in vitro* assay and identification of PDZD8 as a stabilizing cofactor may provide opportunities to better understand the process of capsid uncoating and the specific mechanistic role of PDZD8 in its regulation.

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Chapter Two

Development of an *in vitro* assay of CA-NC stability

Abstract

Following entry into a target cell, the protein capsid of the HIV-1 virus begins disassociation from the viral genome in a process known as uncoating. Cellular factors that affect this process by stabilizing or destabilizing the viral core can have a significant effect on HIV-1 infectivity. We have developed a novel *in vitro* assay of capsid stability using HIV-1 CA-NC complexes that is sensitive to a previously unidentified capsid-stabilizing factor in cytoplasmic lysates. Here, we show that a heat-labile factor, present in the cytoplasm of cells from multiple vertebrate species and greater than 100 kilodaltons in size, slows the spontaneous disassembly of HIV-1 capsid-nucleocapsid (CA-NC) complexes *in vitro*.

Introduction

The human immunodeficiency virus 1 (HIV-1) RNA genome is contained within the viral capsid, a conical protein shell composed of ~1500 molecules of the capsid (CA) protein (13).

The CA proteins of HIV-1 and other retroviruses oligomerize into hexamers by virtue of interactions between the N-terminal domains of CA molecules. Additional interactions involving the C-terminal and N-terminal domains promote inter-hexamer interactions that allow the capsid structure to be assembled (12, 21). Following virus entry into the host cell cytoplasm, the capsid disassembles and CA proteins gradually disassociate from the viral genome, a process called uncoating.

Regulation of the rate of uncoating is critical to successful retroviral infection (5, 9, 23). Alterations in uncoating can affect reverse transcription and the nuclear import of viral cDNA (3, 9, 25). Some changes in CA as well as CA-binding small molecules can alter CA-CA intermolecular interactions and lead to premature viral capsid uncoating; premature uncoating is associated with lower levels of reverse transcription and substantially decreased infectivity (5, 9, 23). Additionally, cellular factors have been shown to affect retroviral infectivity by altering the kinetics of capsid uncoating. The cellular restriction factors TRIM5 α and TRIMCyp increase the rate of capsid disassembly, inhibiting infectivity of susceptible retroviruses (22, 24, 25). Conversely, the cellular cofactor cyclophilin A enhances HIV-1 capsid stability and is required for efficient infection in some cell types (17).

The process of uncoating and the mechanisms by which cellular cofactors regulate uncoating kinetics remain poorly understood. Investigation of HIV-1 capsid stability *in vivo* has proven difficult due to the relatively small percentage of internalized CA protein associated with

infectious cores during the early phase of retroviral infection. Multiple different experimental protocols have been developed to specifically highlight or isolate CA associated with infectious cores for study *in vivo* (1, 3, 14, 20, 25, 26). However, there is significant disagreement within the field regarding the ability of each of these assays to capture representative samples of infectious cores (see chapter 1, section 2.2 for additional discussion of these assays). These dissimilar experimental methodologies have produced seemingly contradictory conclusions regarding capsid uncoating and there is little consensus regarding important elements of uncoating biology including the site of uncoating and the half-life of intracellular viral cores (2, 14).

In vitro assays of capsid uncoating offer the opportunity to bypass some of the challenges inherent in the use of the aforementioned *in vivo* assays. The use of *in vitro* assays eliminate two major sources of CA protein that are not relevant to potential infectious cores (a primary confounding factor in analysis of capsid uncoating *in vivo*): 1) CA in virions that is not assembled in a multimerized capsid, and 2) capsids which are endocytosed rather than released into the cytoplasm by membrane fusion. Additionally, cellular factors hypothesized to influence capsid uncoating kinetics can be isolated or eliminated from *in vitro* assays of uncoating by fractionation methods that are incompatible with the use of live cellular targets in *in vivo* assays.

Previously developed experimental protocols to evaluate retroviral capsid uncoating *in vitro* have made use of delipidated virions as a source of viral capsids (9). These assays have been useful tools in the identification of CA residues critical to capsid stability and in the discovery of a putative cellular active uncoating factor (4, 9). However, the usefulness of these assays, particularly with regard to the study of cellular cofactors of uncoating, has been limited by the inherent instability of the viral cores *in vitro* (9). For this reason, we developed an assay

to study host cell proteins that affect the stability of *in vitro*-assembled HIV-1 capsid-nucleocapsid (CA-NC) complexes. These multimeric assemblies of purified CA-NC protein form cylindrical tubes with a hexameric lattice similar to that of the viral capsid (11, 16). The inclusion of the uncleaved nucleocapsid (NC) region of HIV-1 Gag permits nucleotide-mediated dimerization of the CA-NC protein, resulting in a capsid analog that is more stable than purified viral cores. Due to their capsid-like hexameric lattice and enhanced stability in various experimental conditions, *in vitro* assembled CA-NC complexes have been used in both structural and protein-binding studies in place of the isolated viral capsids (11, 18). We used these CA-NC complexes as substrates in an assay designed to identify components of cytoplasmic extracts that alter the disassembly of the capsid-like lattice.

Here, we present evidence that cytosolic extracts from vertebrate cells, but not bacterial lysates, stabilize CA-NC complexes *in vitro*. Fractionation of the cytoplasmic lysates and characterization of the biochemical properties of the capsid-stabilizing activity indicate that the stabilizing factor is likely a protein with a molecular weight in excess of 100-kDa. The development of an *in vitro* assay that captures the effect of capsid-stabilizing cofactors represents a significant opportunity to advance the study of retroviral uncoating.

Materials and Methods

Cells, plasmids and siRNA. Mammalian and avian cells were grown in Dulbecco's modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin.

Assembly of HIV-1 CA-NC complexes. Purification of recombinant HIV-1 capsid-nucleocapsid (CA-NC) protein from *Escherichia coli* was carried out as previously described (11). High-molecular-weight HIV-1 CA-NC complexes were assembled using 300 μ M CA-NC protein and 60 μ M (TG)₅₀ DNA oligonucleotide in a volume of 100 μ l of 1x PBS and 500 mM NaCl (11). The reaction was allowed to proceed overnight at 4°C. Immediately before use, the assembled CA-NC complexes were spun at 10,000 x g for 1 minute and resuspended in 1x PBS.

Isolation of cytosolic lysate. 250,000 eukaryotic cells were resuspended in 200 μ l 1x PBS and lysed by a 1-minute treatment by a Kontes pestle. Cellular membranes and large complexes were then removed in a two-part centrifugation protocol. First, the lysed material was spun for 1 hour at 15,000 x g at 4°C. Second, the supernatant from the first spin was loaded onto a 3-ml 55% (w/v) sucrose cushion and spun in a Beckman SW55Ti rotor at 115,000 x g for 2 hours at 4°C. Following this centrifugation step, the supernatant above the sucrose layer was removed, quantitated by Coomassie (Bradford) Protein Assay (Pierce) and used in the CA-NC stabilization assay.

To generate *E. coli* whole cell lysate, 2 ml of B-PER bacteria protein extraction reagent (Pierce) was used to resuspend a 0.5 mg pellet of DH5 alpha *E. coli* bacteria. Following resuspension of the pellet, the reagent was incubated for 15 minutes at room temperature and mixed by vortex for 30 seconds. The mixture was then centrifuged at 15,000 x g for 5 minutes to remove insoluble proteins. The supernatant fraction was dialyzed in 1x PBS for 4 hours three times. The dialyzed fraction was then loaded onto a 3-ml 55% (w/v) sucrose cushion and spun in a Beckman SW55Ti rotor at 115,000 x g for 2 hours at 4°C. Following this centrifugation step, the supernatant above the sucrose layer was removed and quantitated as described above.

CA-NC Stabilization Assay. Two μl of assembled CA-NC complexes were added to 250 μl of 293T cell lysate (diluted to a protein concentration of 0.5 mg/ml). The CA-NC complexes and cell lysate were gently mixed at room temperature for 1 hour (unless otherwise indicated). This mixture was layered onto a 3.5-ml 70% sucrose cushion and spun at 110,000 $\times g$ for 60 minutes in an SW55Ti rotor at 4°C. The “Pellet” fraction was then resuspended in 100 μl in 1x SDS running buffer. CA-NC content of this Pellet fraction was determined by SDS-PAGE gel electrophoresis.

An alternate protocol including fractionation of the sucrose cushion was also used. In this protocol, 10 μl of assembled CA-NC complexes were added to 250 μl of cytoplasmic lysate diluted to 0.2 $\mu\text{g}/\text{ml}$ in 1x PBS buffer. The CA-NC complexes and cell lysate were gently mixed at room temperature for the indicated time. This mixture was then layered onto a 3.5-ml 70% sucrose cushion and spun at 50,000 $\times g$ for 20 minutes in an SW55Ti rotor at 4°C. Following centrifugation, 250- μl fractions were removed from the centrifuge tube using a peristaltic pump. A final “Pellet” fraction was created by resuspending any pelleted material in 250 μl of 1x PBS.

The CA-NC content of individual fractions was assayed by ELISA. Sucrose fractions were diluted 1:10 in BupH Carbonate-Bicarbonate Buffer pH9.4 (Pierce). Fifty μl of diluted fractions were then added to white flat-bottom 96-well plates (NUNC) for 1 hour at room temperature. Plates were blocked with 20% FBS in 1x PBS for an additional 1 hour. The plates were then treated with anti-p24 HRP-conjugated antibody (AbCam) at 1 $\mu\text{g}/\text{ml}$ in 1x PBS + 0.05% Tween for 1 hour at room temperature. Plates were washed 2x in blocking buffer and 3 times in 1x PBS + 0.05% Tween 20. HRP levels were then detected by Supersignal Pico Chemiluminescent Substrate (Pierce), using a 96-well plate luminometer in accordance with the

manufacturer's protocols. A series of dilutions of purified CA-NC protein in the binding buffer was included in all plates as a standard to allow quantitation. All fractions were quantitated in duplicate. All experiments are representative of data from at least three experiments.

Results

Cell lysates stabilize HIV-1 CA-NC complexes

The binding of cellular proteins such as TRIM5 α to HIV-1 capsid complexes has previously been studied by examining the co-sedimentation of the host protein with CA-NC complexes assembled *in vitro* (25). During the course of these studies, we noted that a greater percentage of input CA-NC complexes passed through the high-density sucrose cushion following incubation with cytoplasmic lysates from HeLa cells, relative to that seen after incubation in PBS. To explore this finding further, we tracked the sedimentation of *in vitro* assembled CA-NC complexes that were incubated with cell lysates or PBS. The CA-NC complexes were incubated at room temperature in PBS buffer alone or PBS buffer containing cytoplasmic lysates from selected human cell lines for 1 hour before loading onto a 3.5-ml 70% sucrose cushion. Following centrifugation, the pellet fraction was resuspended in 1x SDS buffer and analyzed by SDS-PAGE electrophoresis and Coomassie blue staining. The results confirmed that incubation of the CA-NC complexes in lysates from multiple vertebrate cell lines enhanced the recovery of CA-NC complexes in the pellet fraction (Figure 2.1). Whole cell lysate however did not enhance pelleting of CA-NC.

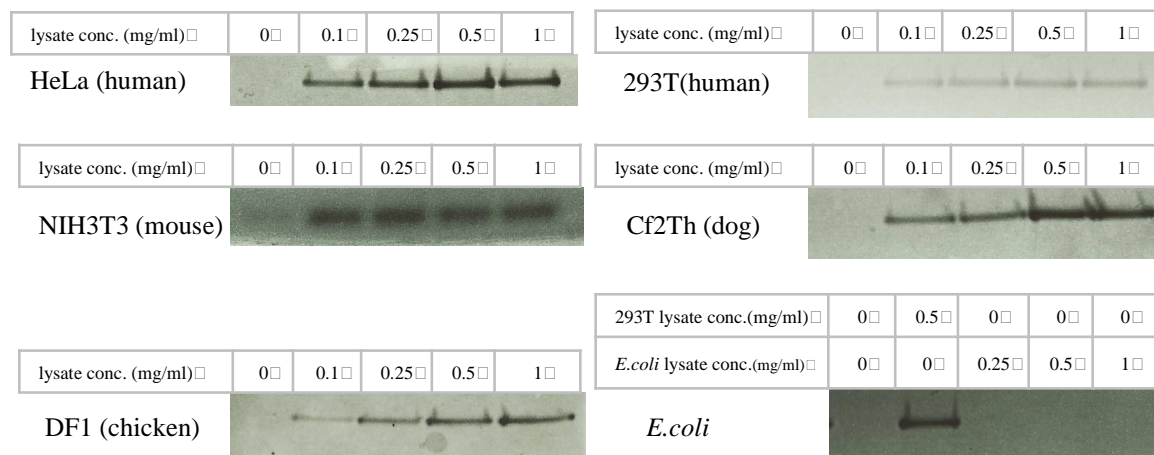


Figure 2.1 Stabilization of CA-NC complexes by lysates from multiple cell types. Two μ l of the *in vitro* assembled HIV-1 CA-NC complexes were gently mixed with 250 μ l cell lysate at the indicated concentrations in 1x PBS or 1x PBS buffer alone for 1 hour at 20°C. The mixture was then loaded onto a 3.5-ml 70% sucrose cushion and spun at 110,000 x g in a Beckman SW55Ti rotor for 60 minutes at 4°C. Following centrifugation, the pellet was resuspended in 100 μ l 1x SDS loading buffer. Pellet fractions were analyzed by SDS-PAGE and stained with Coomassie Blue. The SDS-PAGE gel regions containing CA-NC from the pellet fractions are shown.

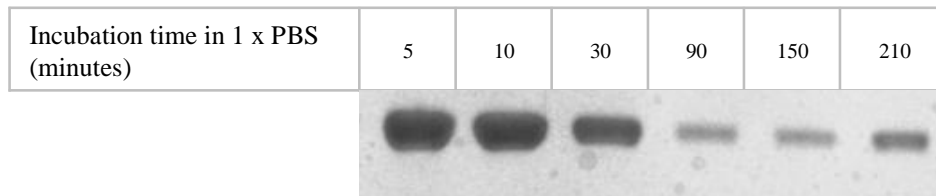
We hypothesized two potential mechanisms of the lysate-dependent enhancement of CA-NC recovery, which we then sought to evaluate experimentally. These are:

(1) *Enhanced CA-NC sedimentation rate by co-sedimentation.* The observed increase in CA-NC recovery is potentially the result of a CA-NC binding component of the cytoplasmic lysate with a higher sedimentation rate (*e.g.* a very large and/or dense complex) that induces rapid sedimentation of bound CA-NC protein through the sucrose cushion during ultracentrifugation

(2) *Enhanced CA-NC stability.* Alternately, a component of the cytoplasmic lysate may serve to slow the disassembly of large, high sedimentation rate CA-NC complexes. Preparations of *in vitro* assembled CA-NC complexes are composed of a heterogeneous mixture of CA-NC tubes of varying sizes (7, 12). The sedimentation rate of these CA-NC complexes is dependent on size with large complexes sedimenting more rapidly. Ultracentrifugation of CA-NC tube

preparations incubated in PBS alone over multiple time points indicates that the amount of high sedimentation rate complexes diminishes over time, consistent with the progressive disassembly of large tubes (Figure 2.2A). A slowed disassembly of large CA-NC tubes would also lead to the increased CA-NC levels in the post-centrifugation pellet fraction that we observed following incubation in cytosolic lysate relative to the CA-NC in the pellet fraction following incubation in buffer alone.

A



B

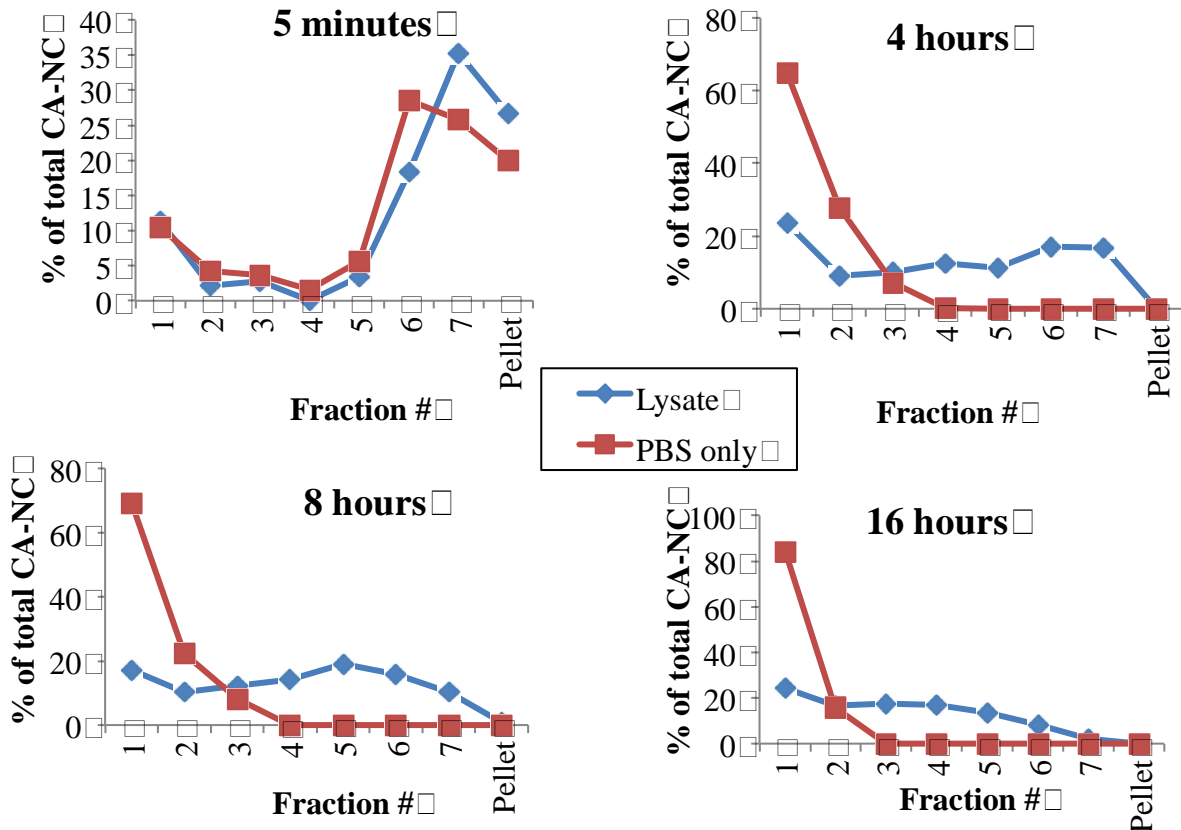


Figure 2.2 Loss of high-sedimentation-rate CA-NC complexes over time. (A) Loss of high-sedimentation-rate complexes. Two μ l of the *in vitro* assembled HIV-1 CA-NC complexes were gently mixed with 1x PBS buffer alone for 5 minutes to 210 minutes as indicated at 20°C. The mixture was then loaded onto a 3.5-ml 70% sucrose cushion and spun at 110,000 \times g in a Beckman SW55Ti rotor for 60 minutes at 4°C. Following centrifugation, the pellet was resuspended in 100 μ l 1x SDS loading buffer. Pellet fractions were analyzed by SDS-PAGE and stained with Coomassie Blue. The SDS-PAGE gel regions containing CA-NC from the pellet fractions are shown. (B) Tracking the stabilization of HIV-1 CA-NC complexes by cellular lysate over a 16-hour time course. Ten μ l of the *in vitro* assembled HIV-1 CA-NC complexes were gently mixed with 250 μ l of HeLa cell lysate (0.2 mg/ml in 1x PBS) or 1x PBS buffer alone for 5 min, 4 hr, 8, or 16 hours at 20°C. The mixture was then loaded onto a 3.5-ml 70% sucrose

Figure 2.2 (continued) cushion and spun at 50,000 x g in a Beckman SW55Ti rotor for 20 minutes at 4°C. Following centrifugation, 500- μ l fractions were taken from the top (250 μ l supernatant included in Fraction 1). The pellet was resuspended in 250 μ l 1x PBS and included in Fraction 8. The CA-NC content of each fraction was determined by ELISA and normalized to the total CA-NC in the sample. These experiments were conducted three times and the results from a typical experiment are shown.

The findings from several experimental approaches have been consistent with the latter hypothesis of enhanced tube stability. First, we determined that incubation of CA-NC tubes in cytoplasmic lysate does not appear to enhance CA-NC sedimentation rate. To test this, we developed an alternate CA-NC stabilization assay protocol designed to more precisely track the sedimentation rates of CA-NC complexes following centrifugation. CA-NC complexes were incubated at room temperature in PBS buffer alone or PBS buffer containing HeLa cell lysate for 5 minutes, 4 hours, 8 hours, or 16 hours before loading onto a 3.5-ml 70% sucrose cushion. After centrifugation at 50,000 x g for 20 minutes, 250- μ l fractions were taken, starting from the top (the supernatant was also saved, and any pelleted material was re-suspended in 250 μ l of 1x PBS). The CA-NC protein within each fraction was quantitated by ELISA (Figure 2.2B). At the early 5-minute time point, CA-NC tubes co-incubated with either 1x PBS or cytoplasmic lysate have similar percentages of high sedimentation rate complexes (those in the higher-numbered fractions). Incubation in the cytoplasmic lysate does not result in any increase in the percentage of high-sedimentation-rate complexes over time. Instead, at later time points, the abundance of high-sedimentation-rate complexes diminish in both lysate and buffer-only conditions with a more rapid loss occurring following incubation in buffer alone. These results are consistent with a tube stabilization model proposing that cytoplasmic lysates stabilize the CA-NC complexes. However, we note that this finding is not sufficient to exclude the possibility that CA-NC tubes are binding quickly sedimenting cellular factors that increase the sedimentation rate of bound

tubes, but that this enhancement of sedimentation rate is counterbalanced by the simultaneous disassembly of those same tubes over time.

Additionally, we sought to determine if the loss of pelletable CA-NC complexes following incubation in buffer alone could be reversed by subsequent incubation in cytoplasmic lysate. To do so, we incubated CA-NC complexes in PBS alone or 293T lysate at concentrations of 0.25 mg/ml or 0.5 mg/ml for 30 minutes. Lysate and 1x PBS was then added to each sample so as to bring the final concentration of 293T lysate in each sample to 0.25 mg/ml. Complexes and lysate were then mixed for an additional 2 hours. Following this second mixing step, CA-NC complexes were loaded over a sucrose cushion and the CA-NC tube stabilization assay was performed as described above (Figure 2.3). Pelletable CA-NC complexes, lost during incubation in buffer alone, were not restored by subsequent incubation in cytoplasmic lysate. This is also consistent with a model in which the active component of the cytoplasmic lysate slows the disassembly of larger CA-NC complexes.

Initial lysate concentration, □ 30 minute incubation (mg/ml) □	0 □	0.5 □	0.25 □	0.5 □	0 □
Final lysate concentration (mg/ml) □ 120 minute incubation □	0 □	0.5 □	0.25 □	0.25 □	0.25 □




Figure 2.3 Pelletable CA-NC loss appears irreversible. Ten μ l of the *in vitro* assembled HIV-1 CA-NC complexes were gently mixed with 150 μ l cell lysate (lysate concentrations in 1x PBS are as listed in the Figure) or 1x PBS buffer alone for 30 minutes at 20°C. An additional 150 μ l cell lysate or PBS was then added to these samples and mixed for another 120 min. 250 μ l of this mixture was then loaded onto a sucrose cushion and the CA-NC stabilization assay was performed. This experiment was conducted 3 times, and the results from a typical experiment are shown.

Finally, to complement these approaches, we attempted to isolate and physically remove any rapidly sedimenting lysate components prior to co-incubation with the CA-NC complexes. We hypothesized that if increased CA-NC pelleting was due to co-sedimentation with rapidly sedimenting CA-NC-binding partners, removal of these factors would abrogate lysate-dependent enhancement of CA-NC sedimentation rate. Rapidly-sedimenting lysate components were isolated by ultracentrifugation. 293T cytoplasmic lysate was loaded onto a 55% sucrose cushion and spun at 100,000 or 200,000 x g for 30 minutes or 1 hour as indicated (Figure 2.4B). Following centrifugation, the supernatant fraction was collected, quantitated, and incubated with CA-NC complexes in a stabilization assay. Incubation of this supernatant fraction stimulated greater recovery of input CA-NC than incubation with an equal concentration of unspun lysate, indicating that the responsible lysate components do not rapidly pass into sucrose cushions in the absence of CA-NC complexes

pre-incubation centrifugation duration (minutes)	0	30	60	60
pre-incubation centrifugation force (1,000 x g)	N/A	100	100	200

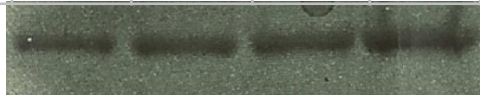


Figure 2.4 Pre-clearance of high-sedimentation-rate complexes prior to the stabilization assay. To remove rapidly sedimenting components of cell lysate, a 293T cytoplasmic lysate was centrifuged prior to co-incubation with CA-NC complexes. The 293T cytoplasmic lysate was loaded onto a 3.5-ml sucrose cushion and spun in a Beckman SW55Ti rotor at 100,000 x g or 200,000 x g for 30 or 60 minutes as indicated. Following centrifugation, the supernatant fraction above the sucrose cushion was collected. The protein content of each supernatant fraction was quantitated and diluted in 1x PBS buffer to reach a protein concentration of 0.2 mg/ml. The diluted lysate was then used in a CA-NC tube stabilization assay. This experiment was conducted 3 times, and the results from a typical experiment are shown

In sum, these findings contradict the hypothesis that lysate-dependent CA-NC pelleting is due to co-sedimentation with a large or dense binding partner. Instead, these data are generally

consistent with the hypothesis that co-incubation of CA-NC tubes with cytosolic lysate slows the disassembly of those tubes. This hypothesis is further bolstered by evidence that we present in chapter 3 of this dissertation that the responsible factor, PDZD8, stabilizes HIV-1 cores *in vivo* as measured by the fate-of-capsid assay. Recently, another group using a similar assay also concluded that cytosolic lysate stabilizes the HIV-1 core *in vitro* (10).

Ionic strength and nucleic acid concentration do not significantly contribute to stabilization

In vitro assembly of concentrated purified CA-NC protein requires both the presence of a high concentration of nucleic acid and high-ionic-strength buffer (7, 18). Because these conditions contribute to the initial formation of the large CA-NC tubes, we sought to determine if the stabilization effect observed from addition of cytoplasmic lysate could be due to either of these factors.

To evaluate the effect of buffer ionic strength on CA-NC stabilization, CA-NC complexes were incubated in 293T lysate dialyzed in 1x PBS buffer, PBS buffer alone, or PBS buffer with elevated NaCl concentrations of 0.5M, 1M and 2M. Incubation in the high-ionic-strength buffers in the absence of cytoplasmic lysate did not stabilize CA-NC complexes (Figure 2.5A).

We also experimentally evaluated the effects of nucleic acid concentration on the stabilization of CA-NC complexes. During our *in vitro* assembly of CA-NC tubes, short ssDNA 50mers are added at a concentration of 2 mg/ml. These ssDNA molecules bind the NC domains of multiple CA-NC molecules, inducing CA-NC dimerization and CA-NC tube formation (7). We sought to determine if the CA-NC- stabilizing effect of cytoplasmic lysate might be due to the presence of additional nucleic acid. Incubation of CA-NC complexes in PBS with a

concentration of ssDNA as high as 2 mg/ml did not stabilize CA-NC tubes (2.5B). Additionally, pretreatment of cytoplasmic lysate with the nuclease Benzonase did not diminish the CA-NC-stabilizing effect of lysate (Figure 2.5C).

Our data indicate that the observed CA-NC stabilization effect is unlikely to be due to the ionic strength or the nucleic acid concentration of the cytoplasmic lysate.



Figure 2.5 The effect of NaCl and nucleic acid concentration on CA-NC stabilization. (A) Effect of elevated NaCl concentration on stabilization of CA-NC complexes. Two μ l of the *in vitro* assembled HIV-1 CA-NC complexes were gently mixed with 250 μ l 0.5 mg/ml 293T cell lysate in 1x PBS, 1x PBS buffer alone, or 1x PBS buffer with additional NaCl added as indicated for 1 hour at 20°C. A stabilization assay was then performed as described in Figure 2.1. (B) Effect of ssDNA on the ability of 1x PBS buffer to stabilize HIV-1 CA-NC complexes. 2 μ l of CA-NC complexes were incubated with 293T cell lysate at 0.5 mg/ml, 1x PBS buffer alone, or 1x PBS with indicated concentration of ssDNA (a 50-mer of alternating T- and G- bases) at 20°C for 1 hour. (C) Effect of Benzonase treatment on the HIV-1 CA-NC-stabilizing activity. Prior to use in the stabilization assay, 293T lysates were incubated at 20° or 37° C with or without 50 U/ml of Benzonase nuclease. Following incubation, lysates were brought back to room

Figure 2.5 (continued) temperature and used in a CA-NC stabilization assay. All experiments were performed three times and a representative example is presented.

Stabilization by protein component of cytoplasmic lysate


The results of several experiments are consistent with the hypothesis that a protein factor is responsible for the stabilization of CA-NC complexes by cytosolic lysates. To isolate the protein fraction of the cytoplasmic lysate, whole lysate was precipitated with concentrated ammonium sulfate (15). At 50% concentrated ammonium sulfate, but not at 33% concentrated ammonium sulfate, the stabilizing component of 293T cell cytoplasmic lysate is precipitated from solution (Figure 2.6A). The stabilizing effect of this component can be subsequently reconstituted by resuspension of precipitated material in PBS. Precipitation at this concentration of ammonium sulfate is characteristic of a protein factor (15).

To test whether the stabilizing component of lysate was heat-labile, we incubated 293T lysate in 37°C for periods of up to 2 hours (Figure 2.6B). Following incubation at 37°C, the lysates were returned to room temperature and used in CA-NC stabilization assays. The CA-NC-stabilizing effect of lysate diminished with increasing time at 37°C.

We also examined whether the stabilizing effect might be due to a non-specific crowding effect of protein. The stabilization of CA-NC by cytoplasmic lysate is dependent on protein concentration, but does not appear to be a non-specific property of all proteins. Co-incubation of CA-NC with high concentrations of BSA and FBS dialyzed in PBS did not stabilize CA-NC complexes to a level comparable to that achieved by co-incubation with cytoplasmic lysate (Figure 2.6C). The stabilizing effect appears to be the result of specific protein factor or factors contained within the cytoplasmic lysate.

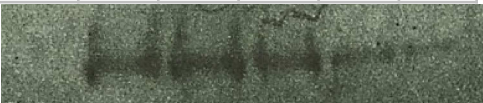
A

saturated NH_4SO_4 □ (% of total volume) □	0 □	33 □	33 □	50 □	50 □	66 □	66 □
soluble (S) or pellet (P) □	S □	P □	S □	P □	S □	P □	S □



B

	PBS	293T lysate (0.5 mg/ml)				
pre-incubation at 37°C (min)	N/A	0	15	30	60	120



C

PBS	Lysate (0.5 mg/ml)	Lysate (0.25 mg/ml)	BSA (2 mg/ml)	FBS (4 mg/ml)
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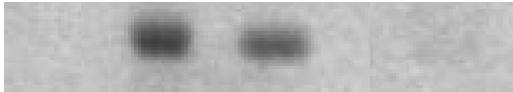


Figure 2.6 Characterization of the HIV-1 CA-NC complex-stabilizing factor in cell lysates. **(A)** Stabilization of CA-NC complexes by ammonium sulfate-precipitated lysate. One ml of 293T lysate (3 mg/ml) in 1x PBS + 1M NaCl was mixed with concentrated ammonium sulfate in filtered H_2O such that the concentrated ammonium sulfate solution was 33%, 50% or 66% of the final volume. The samples were then mixed at 4°C for 4 hr. Precipitated protein was isolated by centrifugation at 15,000 x g for 30 min. Any pelleted material was resuspended in 250 μl 1x PBS buffer. Both resuspended pellet (P) and supernatant (S) fractions were dialyzed in 1x PBS for 2 hours 3 times. Following dialysis, the protein content of each fraction was quantitated and each fraction was diluted in 1x PBS to a concentration of 0.5 mg/ml. Finally 250 μl of each fraction was then used in a CA-NC stabilization assay. **(B)** Effect of preincubation at 37°C on the ability of a 293T cell lysate to stabilize HIV-1 CA-NC complexes. A 293T cell lysate at 0.5 mg/ml was incubated at 37° C for the indicated times. Following incubation, lysates were brought back to room temperature and used in a CA-NC stabilization assay. **(C)** Comparison of CA-NC stabilization by a 293T lysate and purified bovine serum albumin (BSA) and filtered fetal bovine serum (FBS) at the indicated concentrations. Filtered 2 mg/ml milk solution also failed to stabilize CA-NC complexes (data not shown). All of these experiments were performed three times and typical results are shown.

Assessing the size of the stabilizing factor(s)

To facilitate identification of those factors primarily responsible for the CA-NC-stabilizing effect, we attempted to isolate the active factor or factors by size. To do so, we fractionated a cytoplasmic lysate by both filtration and size-exclusion chromatography. Centrifugal filtration indicated that no significantly stabilizing component of the lysate passed through filters with nominal molecular weight limits (NMWL) of <100-kDa (Figure 2.7A).

Separation by filters with 300-kDa NMWL resulted in stabilization by both the flow-through and retained fractions. This may indicate the presence of multiple stabilizing components or that the stabilizing component exists in lysates in multimers, complexes, or degradation products of multiple sizes.

In addition to centrifugal filtration, stabilizing lysates were also separated by size-exclusion chromatography over Superdex 200 column (GE Healthcare). Following size-exclusion fractionation, individual fractions were dialyzed in 1x PBS buffer and assayed for CA-NC-stabilizing activity. The relative size of each fraction's component proteins was estimated by comparison to a standard ladder. CA-NC tubes were most stabilized by fractions corresponding to sizes 75-125-kDa, 300-kDa, and the void fraction (containing lysate components estimated to be >600-kDa) (Figure 2.7B).

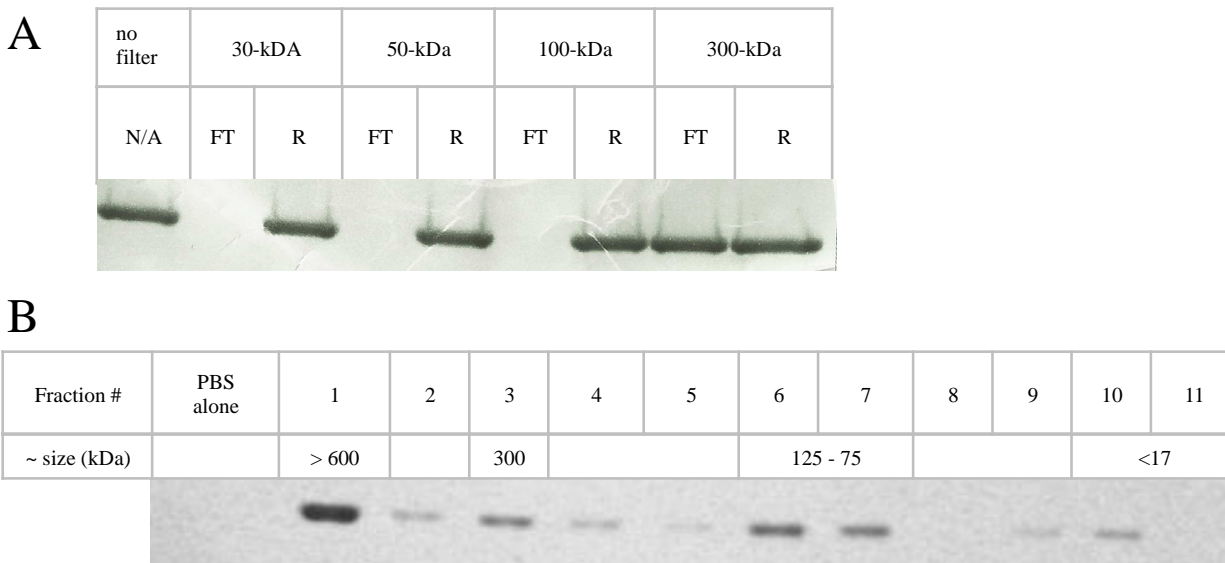


Figure 2.7 Assessing the size of the HIV-1 CA-NC complex-stabilizing factor in cell lysates. (A) Stabilization of HIV-1 CA-NC complexes by 293T cell lysate fractions. Following filtration of 0.5 mg/ml 293T cell lysate through filters of the indicated molecular weight cut offs (30-kDa, 50-kDa, 100-kDa, Millipore; 300-kDa, Sigma-Aldrich), the retained (R) and flow-through (FT) fractions were used in a CA-NC stabilization assay. (B) Fractionation of lysate by size-exclusion chromatography. 0.5 ml of 3 mg/ml 293T cytoplasmic lysate was loaded onto a Superdex 200 5/150GL column. Following sample loading, 1x PBS was run over the column and 250 μ l fractions were collected. Absorbance at 280 nm indicated the presence of protein in 11

Figure 2.7 (continued) consecutive fractions. Each of these fractions was assayed for protein concentration and diluted in 1x PBS to a concentration of 0.1 mg/ml. Finally, 250 μ l of each diluted fraction was then used in a CA-NC stabilization assay. A mixture of known protein standards was run under identical conditions to estimate the size of protein components in each fraction. The approximate size of proteins in stabilizing fractions as determined by comparison to these standards is also shown. These experiments were conducted three times and the results from typical experiments are shown.

Discussion

Decreases in the stability of retroviral capsids have substantial negative impacts on viral infectivity, typically resulting in defective reverse transcription (9, 25). The retroviral capsid is a loose assemblage of ~1500 CA proteins and exists within the viral membrane in equilibrium with free CA monomers (6). Upon removal of the viral membrane, retroviral capsids are unstable in physiological buffers (9). These observations contrast with the results of fate-of-capsid assays in infected cells, where particulate retroviral capsids exhibit half-lives of 8-10 hours (7, B. A. Bollman, unpublished data). The existence of capsid-stabilizing factors in the cytoplasm of vertebrate host cells provides a solution to the contrasting behavior of retroviral capsids *in vitro* and in infected cells.

Investigation of cellular factors that regulate the uncoating kinetics of retroviruses is an active field of research. Cellular proteins that enhance capsid disassembly as well as one factor thought to slow capsid uncoating have been identified as restriction factors that inhibit HIV-1 infectivity (8, 22, 24, 25, 27). Prior to this research, only cyclophilin A had been identified as positive cofactor of HIV-1 infectivity that enhances viral capsid stability (17). Cyclophilin A does not appear to be the factor responsible for stabilization of CA-NC tubes *in vitro*. Size fractionation of lysate by centrifugal filtration units and size-exclusion chromatography remove nearly all detectable levels of cyclophilin A from lysate fractions that retain stabilization activity

(data not shown). The data presented here strongly indicate the existence of at least one additional stabilizing component present in human cell cytoplasm.

The discovery of a previously unknown CA-NC-stabilizing component of cytoplasmic lysate stimulated our investigation into the identity of specific stabilizing factors (discussed in chapter 3). The development of this *in vitro* CA-NC assay was of significant utility in this investigation as it, unlike previously developed *in vivo* assays of uncoating, permitted analysis of lysate which has been fractionated to isolate active components. We believe this assay has the potential to be used in the isolation of additional capsid-regulating factors by a similar methodology.

The study of cellular regulators of viral capsid stability are of particular interest because the process of viral uncoating is so poorly understood. The mechanisms by which these factors alter capsid stability are currently unknown (19). The development of an *in vitro* assay of CA-NC stability offers an opportunity to experimentally investigate the mechanism of capsid stabilization by methods incompatible with previously described experimental protocol. *In vivo* assays of stabilization, like the fate-of-capsid assay, are limited by the need for viable cell targets. Under these conditions, isolation of specific factors of interest is challenging or impractical. The development of this *in vitro* assay could be used in future work to investigate the stabilizing factor that we have observed here, as well as other known regulators of capsid stabilization. Such studies can focus on the precise mechanisms of capsid and factor regulation as well as the relationship of these elements to each other.

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Chapter Three

Cellular factor PDZD8 contributes to the stabilization of
the HIV-1 capsid

Abstract

Following human immunodeficiency virus (HIV-1) entry into the host cell, the viral capsid gradually disassembles in a process called uncoating. A proper rate of uncoating is important for reverse transcription of the HIV-1 genome. Host restriction factors such as TRIM5 α and TRIMCyp bind retroviral capsids and cause premature disassembly, leading to blocks in reverse transcription. Other host factors, such as cyclophilin A, stabilize the HIV-1 capsid and are required for efficient infection in some cell types. We identified the PDZ-Domain-containing protein 8 (PDZD8) as a critical component of the capsid-stabilizing activity in the cytoplasmic extracts. PDZD8 has been previously reported to bind the HIV-1 Gag polyprotein and to make a positive contribution to the efficiency of HIV-1 infection (17). PDZD8 knockdown accelerated the disassembly of HIV-1 capsids in infected cells, resulting in decreased reverse transcription. The PDZD8 coiled-coil domain is sufficient for HIV-1 capsid binding, but other parts of the protein, including the PDZ domain, are apparently required for stabilizing the capsid and supporting HIV-1 infection. In summary, PDZD8 interacts with and stabilizes the HIV-1 capsid and thus represents a potentially targetable host cofactor for HIV-1 infection.

Introduction

Over the past decade, considerable evidence has emerged indicating that the stability of the capsid is dependent on components of the target cell cytoplasm. The cellular restriction factors TRIM5 α and TRIMCyp have been shown to inhibit infection of multiple retroviruses by binding the capsid and inducing premature uncoating (7, 37, 41). Conversely, when CPSF6, which is normally located in the nucleus, is aberrantly localized in the cytoplasm, the uncoating of some retroviral capsids is slowed, resulting in decreased infection (24). Some of the inhibitory effects on HIV-1 infection associated with knockdown of TNP03, a nuclear import factor, may be due to secondary effects on CPSF6 localization (6, 12, 38). Other cellular proteins affecting the kinetics of retroviral uncoating are positive cofactors of infection. Cyclophilin A, a CA-binding prolyl isomerase, stabilizes the HIV-1 capsid and is required for efficient infection in some cell types (16, 27). As-yet-unidentified host cell factors have been proposed to activate HIV-1 uncoating and induce reverse transcription *in vitro* (3). The mechanisms by which these cellular factors affect capsid stability and the relative contributions of different host factors to retroviral uncoating are under investigation.

Investigation of cellular cofactors of HIV-1 capsid stability and uncoating has proven particularly difficult due to the inherent instability of the viral cores *in vitro*, as well as the relatively small percentage of internalized CA protein associated with infectious cores during the early phase of retroviral infection (1). For this reason, we developed an assay to study host cell proteins that affect the stability of *in vitro*-assembled HIV-1 capsid-nucleocapsid (CA-NC) complexes. These multimeric assemblies of purified CA-NC protein form cylindrical tubes with a hexameric lattice similar to that of the viral capsid (13, 25). The inclusion of the uncleaved nucleocapsid (NC) region of HIV-1 Gag permits oligonucleotide-mediated dimerization of the

CA-NC protein, resulting in a capsid analog that is more stable than purified viral cores. Due to their capsid-like hexameric lattice and enhanced stability in various experimental conditions, *in vitro* assembled CA-NC complexes have been used in both structural and protein-binding studies in place of the natural viral capsid (13, 28). We employed these CA-NC complexes as substrates in an assay designed to identify components of host cell cytoplasmic extracts that altered the disassembly of the capsid-like lattice.

In the previous chapter, we demonstrated that cytosolic extracts from vertebrate cells, but not bacterial lysates, stabilized HIV-1 CA-NC complexes, allowing them to sediment more efficiently through a sucrose cushion. Fractionation of the cytoplasmic lysates and characterization of the biochemical properties of the capsid-stabilizing activity implicated a protein factor of greater than 100-kD molecular weight. Our analysis of candidate capsid-binding proteins identified the PDZ Domain-containing 8 protein (PDZD8) as a critical component of the capsid-stabilizing activity in the cytoplasmic extracts. Human PDZD8 has been previously identified as an HIV-1 cofactor, although its precise role in infection is unknown (17). PDZD8 coprecipitates with the HIV-1 Gag polyprotein and knockdown of the PDZD8 protein decreases HIV-1 infectivity. Additionally, overexpression of PDZD8 has been reported to enhance infectivity of murine leukemia virus (MLV) and simian immunodeficiency virus (SIV) (17). The cellular function of PDZD8 is uncertain, although PDZD8 has been identified as a moesin-interacting factor as well as a potential regulator of microtubule stability (18).

Here, we present evidence that PDZD8 acts to stabilize HIV-1 capsids. Knockdown of PDZD8 by siRNA diminishes the ability of cytoplasmic lysates from human cells to stabilize HIV-1 CA-NC complexes *in vitro*. PDZD8 knockdown accelerates the disassembly of HIV-1

capsids in infected cells, resulting in a block to infection prior to reverse transcription. We examined the ability of PDZD8 variants to bind the HIV-1 CA-NC capsid complexes, identified the PDZD8 coiled-coil as the capsid-binding domain, and showed that capsid binding is not sufficient for stabilization of the CA-NC capsid complexes and enhancement of HIV-1 infectivity. The identification of PDZD8 as a capsid-stabilizing host factor represents a significant opportunity to advance our understanding of the retrovirus uncoating process.

Materials and methods

Cells, plasmids and siRNA. HeLa cells were grown in Dulbecco's modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Prior to transfection, 10,000 HeLa cells were seeded per well in a 24-well plate.

All of the studies reported herein were conducted with human PDZD8. FLAG-tagged wild-type PDZD8 and PDZD8 mutants were cloned in the pIRES2 DsRed2 vector (Clontech). PDZD8-expressing constructs were altered by QuikChange mutagenesis to include silent mutations that prevent targeting by the tested siRNA constructs. Twenty-four hours after seeding, cells were transfected with 0.2 µg plasmid DNA/well with Effectene Transfection Reagent (Qiagen), as described in the manufacturer's protocol.

For siRNA treatment, cells were transfected 24 hours after the Effectene-mediated plasmid transfection with siRNA 21mers siPDZD8#1 (Ambion, s42265) or siPDZD8#2 (Ambion, s42267). The siRNA was transfected at 20 pmol/well with RNAiMAX (Invitrogen), as described in the manufacturer's protocol.

Virus production and infectivity. Retrovirus infectivity assays were carried out with single-round, VSVG-pseudotyped viruses carrying luciferase reporter genes. The pseudotyped HIV-1, simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), Moloney murine leukemia virus (Mo-MLV), and Rous sarcoma virus vectors expressing luciferase were produced in DF1 cells (RSV only) or 293T cells (all other viruses) by co-transfection with a VSV G expression vector, as previously described (5, 29, 31, 34, 35, 40, 44). HIV-1 was concentrated by centrifugation and analyzed by an exogenous ³²P-reverse transcriptase (RT) assay (43). For infections, HeLa target cells were transfected with siRNAs and/or plasmids and then reseeded

into 96-well plates at 6,000 cells/well 24 hours prior to infection. Cells were incubated with pseudotyped viruses at various concentrations, as described in Results. After 4 hours of virus-cell incubation, the virus-containing medium was removed and replaced with fresh medium. Forty-eight hours later, cells were lysed in 30 μ l passive lysis buffer (Promega) and the levels of luciferase activity were assayed, as previously described (33). All infection assays were performed in triplicate.

Measurement of HIV-1 early reverse transcription products. One day prior to infection, 4×10^5 transfected HeLa cells were seeded into 6-well plates in triplicate. VSV G-pseudotyped HIV-1 viruses were prepared as described above and were treated with 44 U/ml of Turbo DNase I (Ambion) at 37°C for 1 hour. After four hours of virus-cell incubation, cells were harvested and total DNA was extracted with the DNeasy kit (Qiagen). One-hundred ng of total DNA was used as a template for quantitative real-time PCR, using previously described conditions (8). Duplicate measurements were performed for each of the triplicate samples.

Assay for stabilization of HIV-1 CA-NC complexes. Purification of recombinant HIV-1 capsid-nucleocapsid (CA-NC) protein from *Escherichia coli* was carried out as previously described (13). High-molecular-weight HIV-1 CA-NC complexes were assembled using 300 μ M CA-NC protein and 60 μ M (TG)₅₀ DNA oligonucleotide in a volume of 100 μ l of 1x PBS and 500 mM NaCl (13). The reaction was allowed to proceed overnight at 4°C. Immediately before use, the assembled CA-NC complexes were spun at 10,000 x g for 1 minute and resuspended in 1x PBS.

As the source of cellular lysate to assay for CA-NC stabilization, HeLa cells were transfected as described above. Forty-eight hours after transfection, 250,000 HeLa cells were resuspended in 200 μ l 1x PBS and lysed by a 1-min treatment by a Kontes pestle. Cellular membranes and large complexes were then removed in a two-part centrifugation protocol. First, the lysed material was spun for 1 hour at 15,000 $\times g$ at 4°C. Second, the supernatant from the first spin was loaded onto a 3-ml 55% (w/v) sucrose cushion and spun in a Beckman SW55Ti rotor at 115,000 $\times g$ for 2 hours at 4°C. Following this centrifugation step, the supernatant above the sucrose layer was removed, quantitated by Coomassie (Bradford) Protein Assay (Pierce) and used in the CA-NC stabilization assay.

In the CA-NC stabilization assay, 10 μ l of assembled CA-NC complexes was added to 250 μ l of HeLa cell lysate (diluted to a protein concentration of 0.2 mg/ml). The CA-NC complexes and cell lysate were gently mixed at room temperature for 4 hours (unless otherwise indicated). This mixture was then layered onto a 3.5-ml 70% sucrose cushion and spun at 50,000 $\times g$ for 20 minutes in an SW55Ti rotor at 4°C. Following centrifugation, 250- μ l fractions (unless otherwise noted in Results) were removed from the centrifuge tube using a peristaltic pump. A final “Pellet” fraction was created by resuspending any pelleted material in 250 μ l of 1x PBS.

The CA-NC content of individual fractions was assayed by ELISA. Sucrose fractions were diluted 1:10 in BupH Carbonate-Bicarbonate Buffer PH9.4 (Pierce). Fifty μ l of diluted fractions was then added to white flat-bottom 96-well plates (NUNC) for 1 hour at room temperature. Plates were blocked with 20% FBS in 1x PBS for an additional 1 hour. The plates were then treated with anti-p24 HRP-conjugated antibody (AbCam) at 1 μ g/ml in 1x PBS + 0.05% Tween for 1 hour at room temperature. Plates were washed 2x in blocking buffer and 3 times in 1x PBS + 0.05% Tween 20. HRP levels were then detected by Supersignal Pico

Chemiluminescent Substrate (Pierce), using a 96-well plate luminometer in accordance with the manufacturer's protocol. Known concentrations of CA-NC protein were included in the binding buffer in all plates to generate standard curves for quantitation. All fractions were quantitated in duplicate. The results reported are representative of data obtained from at least three independent experiments.

PDZD8 binding to HIV-1 CA-NC complexes. Binding of FLAG-tagged PDZD8 variants to HIV-1 CA-NC complexes was assessed with a protocol that uses the assembled CA-NC complexes and HeLa cell lysates described above. In the binding assay, 10 μ l of assembled HIV-1 CA-NC complexes and HeLa cell lysates containing FLAG-tagged PDZD8 variants were gently mixed for 1 hour at room temperature. The mixture was then layered onto a 3.5-ml 70% sucrose cushion and centrifuged at 110,000 \times g for 2 hours at 4° in an SW55Ti rotor. The pellet fraction was resuspended in 100 μ l of sodium dodecyl sulfate (SDS) sample buffer. This sample was then electrophoresed on a polyacrylamide gel, which was used for detection of CA-NC protein by Coomassie Blue staining and FLAG-tagged protein by western blotting, as previously described (28).

The fate-of-capsid assay. Approximately 8×10^5 transfected HeLa cells/well were plated in 6-well plates. On the following day, the cells were incubated with VSV G-pseudotyped HIV-1 virus (5×10^5 RT counts) for 30 minutes at 4°C. Then, the cells were returned to a 37°C CO₂ incubator until they were harvested 16 hours later. The virus suspension was removed at the 4-hour time point and replaced with fresh medium. The cells were washed three times with ice-cold PBS and detached by incubating with 1 ml of pronase (7 mg/ml in DMEM) for 5 minutes at

4°C. The cells were washed once in DMEM containing 10% FBS and twice in PBS. The washed cell pellet was resuspended in 250 µl hypotonic lysis buffer and placed on ice for 15 minutes. The cells were lysed by 1 minute of treatment with a Kontes pestle. Cell debris was removed by centrifugation for 3 minutes at $2,000 \times g$. After centrifugation, 2 ml of lysate was layered onto a 7-ml 50% sucrose cushion (made in PBS) and centrifuged at $125,000 \times g$ for 2 hours at 4°C in a Beckman SW41 rotor. After centrifugation, 100 µl from the top-most part of the supernatant was collected and made 1x in SDS sample buffer. The pellet was resuspended in 100 µl of 1x SDS sample buffer. The samples were subjected to SDS/PAGE and western blotting to detect the capsid proteins.

Results

Stabilization of HIV-1 CA-NC complexes by cell lysates.

The binding of cellular proteins such as TRIM5 α to HIV-1 capsid complexes has previously been studied by examining the co-sedimentation of the host protein with CA-NC complexes assembled *in vitro* (41). During the course of these studies, we noted that a greater percentage of input CA-NC complexes passed through the high-density sucrose cushion following incubation with cytoplasmic lysates from a variety of vertebrate cells, relative to that seen after incubation in PBS. To explore this finding further, we tracked the sedimentation of *in vitro* assembled CA-NC complexes that were incubated with cell lysates or PBS. The CA-NC complexes were incubated at room temperature in PBS buffer alone or PBS buffer containing HeLa cell lysate for either 5 minutes or 4 hours before loading onto a 3.5-ml 70% sucrose cushion. After centrifugation at 50,000 $\times g$ for 20 minutes, 500-ml fractions were taken, starting from the top (the supernatant was included in fraction 1, and any pelleted material was resuspended in fraction 8). The CA-NC protein was detected by western blotting with anti-p24 antibodies (Figure 3.1A). After an incubation of 5 minutes, HIV-1 CA-NC complexes incubated with the HeLa cell lysate sedimented through the sucrose cushion at a rate similar to that of the CA-NC complexes incubated in PBS. By contrast, after a four-hour incubation, rapidly sedimenting CA-NC complexes were much more abundant after incubation in the cell lysate compared with PBS. To quantify this effect, CA-NC levels within fractions taken from the sucrose cushion were determined by ELISA (Figure 3.1B). For these experiments, smaller 250- μ l fractions of the sucrose cushion were collected, with the resuspended pellet kept separate (in Fraction 8) from the supernatant fractions. These results confirmed that a 4-hour incubation with

HeLa cell lysates resulted in faster sedimenting CA-NC complexes than a parallel incubation in PBS (Figure 3.1B).

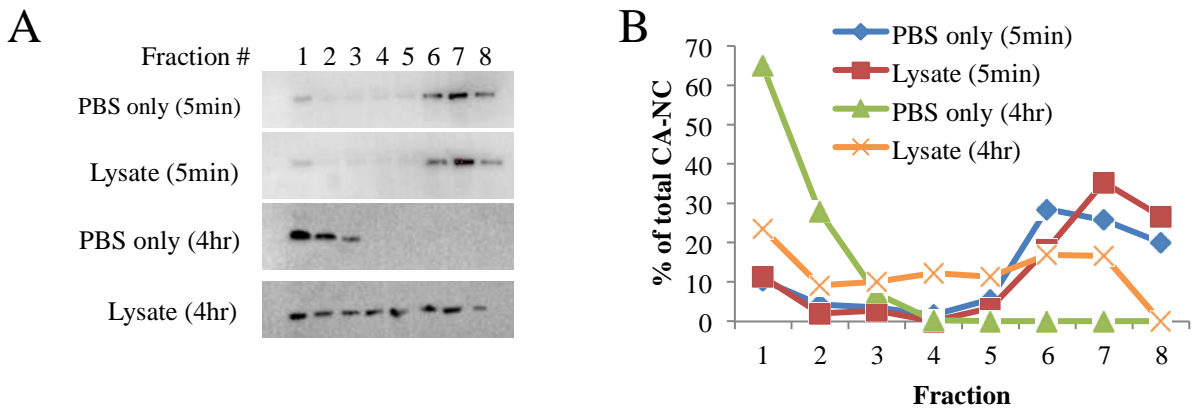


Figure 3.1 Stabilization of HIV-1 CA-NC complexes by cellular lysate. Ten μ l of the *in vitro* assembled HIV-1 CA-NC complexes were gently mixed with 250 μ l of HeLa cell lysate (0.2 mg/ml in 1x PBS) or 1x PBS buffer alone for 5 minutes or 4 hours at 20°C. The mixture was then loaded onto a 3.5-ml 70% sucrose cushion and spun at 50,000 \times g in a Beckman SW55Ti rotor for 20 minutes at 4°C. Following centrifugation, 500- μ l fractions were taken from the top (250 μ l supernatant included in Fraction 1). The pellet was resuspended in 250 μ l 1x PBS and included in Fraction 8. (A) Western blot of fractions with anti-p24-HRP conjugated antibody (Abcam). (B) The CA-NC content of each fraction was determined by ELISA and normalized to the total CA-NC in the sample. This experiment was conducted three times and the results from a typical experiment are shown.

The CA-NC-stabilizing activity was detected in cells of several vertebrate species (human, mouse, dog, chicken), but not in *E. coli*. Analysis of the cell lysate suggested that the factor responsible for the stabilization of HIV-1 CA-NC complexes is heat-labile, nuclease resistant and >100-kDa (see chapter 2). The stabilizing effect was protein content-dependent, but was not observed with equal protein concentrations of dialyzed BSA, dialyzed FBS, or rehydrated milk in PBS. The loss of rapidly sedimenting CA-NC complexes over time is apparently irreversible; incubation of slowly sedimenting CA-NC complexes with cellular lysate did not restore a high sedimentation rate. Together, this evidence suggests that specific components of cytoplasmic lysates from vertebrate cells slow the disassembly of HIV-1 CA-NC

complexes *in vitro*. A recent publication reached a similar conclusion about the ability of human cell lysates to stabilize HIV-1 CA-NC cores (11).

Contribution of PDZD8 to the *in vitro* stabilization of HIV-1 CA-NC complexes.

Human PDZD8, an approximately 160-kD cytoplasmic protein of unknown cellular function, has been shown to bind HIV-1 Gag proteins and to enhance viral infectivity (17). Overexpression of PDZD8 in some cell types has been reported to enhance the production of HIV-1 reverse transcription products (17), indicating a potential role in early post-fusion events in the retroviral life cycle. We hypothesized that PDZD8 might be a retroviral capsid-stabilizing factor, and evaluated PDZD8 as a potential contributor to the *in vitro* stabilization of HIV-1 CA-NC complexes by cell lysates. Transfection with two different siRNAs targeting PDZD8 decreased the capacity of HeLa cell lysates to stabilize HIV-1 CA-NC complexes (Figure 3.2A). Add-back of PDZD8 by transfection with an siRNA-resistant plasmid expressing an N-terminally FLAG-tagged PDZD8 restored the CA-NC-stabilizing ability (Figure 3.2B). Lysate from HeLa cells treated with PDZD8-targeting siRNAs stabilized CA-NC complexes more than PBS alone, but a substantial loss of the fastest sedimenting CA-NC complexes was evident relative to the results obtained with lysate from cells treated with a negative control siRNA. This may reflect incomplete knockdown of PDZD8 expression by the siRNAs used, or may be due to the presence of other CA-NC-stabilizing factors in the cell lysate. Nonetheless, the results suggest that PDZD8 is an important contributor to the *in vitro* HIV-1 CA-NC-stabilizing activity in cell lysates.

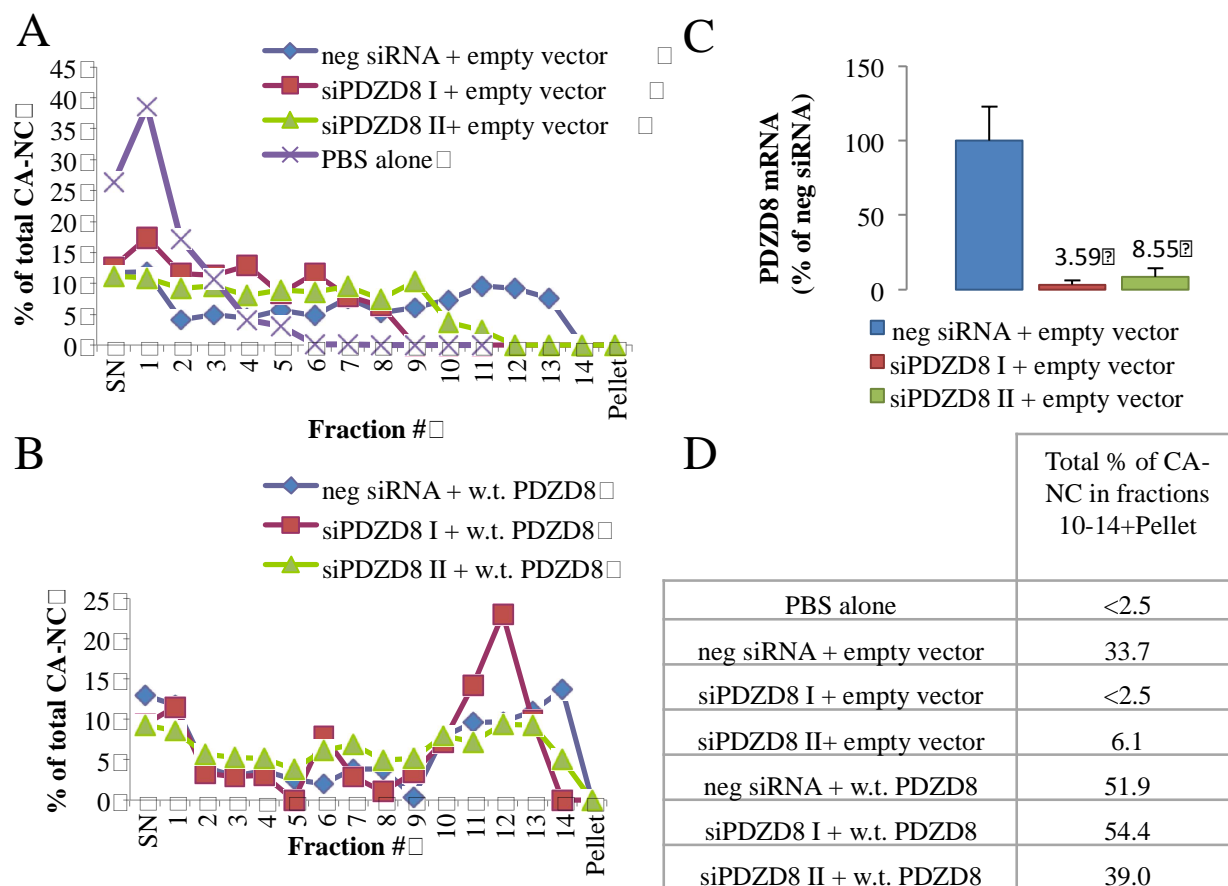


Figure 3.2 PDZD8 contributes to the stabilization of HIV-1 CA-NC complexes *in vitro*. HIV-1 CA-NC stabilization was assayed as described in the Figure 3.1 legend, with the exception that 250- μ l fractions were taken (including a supernatant fraction (SN) and a resuspended pellet fraction). HIV-1 CA-NC complexes and HeLa lysates were mixed for 4 hours at room temperature before centrifugation over a 70% sucrose cushion. The HeLa cells that served as sources for the lysates were transfected with either the empty pIRES2 vector (A) or pIRES2 expressing FLAG-tagged wild-type (w.t.) PDZD8 (B). After 24 hr, cells were transfected a second time with either a negative-control noncoding siRNA or one of two PDZD8-targeting siRNAs (siPDZD8 I or siPDZD8 II). Cells were lysed 48 hours after siRNA transfection, and the cell lysates were used in the HIV-1 CA-NC stabilization assay. Following the centrifugation of the lysate-CA-NC mixtures, the CA-NC content of individual fractions was determined by ELISA and normalized to the total CA-NC in each sample. (C) The efficiency of PDZD8 mRNA knockdown by siRNA transfection was assessed by quantitative RT-PCR. The relative abundance of PDZD8 mRNA compared to that in cells treated with a negative control siRNA is shown. (D) The total percentage of CA-NC contained in Fractions 10-14 plus the resuspended pellet fraction is shown for all samples. Data from a control stabilization assay in which 1x PBS alone was used in place of the HeLa lysate is also included. These experiments were conducted three times and the results from a typical experiment are shown.

Contribution of PDZD8 to the stabilization of HIV-1 capsids in infected cells.

We hypothesized that PDZD8 plays a role in stabilizing the HIV-1 capsid that is introduced into the cytosol following virus entry into the host cell. To test this hypothesis, we utilized the previously described fate-of-capsid assay (41). Fate-of-capsid assays have been used to demonstrate increased rates of viral disassembly that are a consequence of viral restriction factors, small molecule inhibitors of infection, and destabilizing changes in the HIV-1 CA-NC protein (27, 39, 41). VSV G-pseudotyped HIV-1 virus was added to HeLa cells transfected with both the PDZD8-silencing and control siRNAs, as described above. Infected cells were lysed 16 hours after infection, and particulate HIV-1 cores were isolated by centrifugation through a 50% sucrose cushion. Knockdown of PDZD8 by specific siRNA reduced the levels of pelletable core relative to the levels seen in cells transfected with the negative control siRNA (Figure 3.3). Add-back of FLAG-tagged wild-type PDZD8 by transfection with an siRNA-resistant expression plasmid restored the level of particulate cores that pelleted through the sucrose cushion. Notably, whereas overexpression of the putative hyperstabilizing restriction factor CPSF6 has been reported to increase the levels of particulate HIV-1 cores in this fate-of-capsid assay (6, 12), expression of exogenous PDZD8 in the control siRNA-transfected cells did not substantially increase core recovery relative to empty vector-transfected controls. A PDZD8 mutant (PDZD8(1-1028)), which contains a C-terminal truncation that has been reported to result in a loss of HIV-1 Gag binding (17), did not restore the stability of the HIV-1 core in the HIV-1-infected cells in which endogenous PDZD8 expression had been knocked down; we show below that the PDZD8(1-1028) mutant lacks the ability to stabilize HIV-1 CA-NC complexes *in vitro*. These results indicate that wild-type PDZD8 contributes to the stability of the HIV-1 capsid in infected cells.

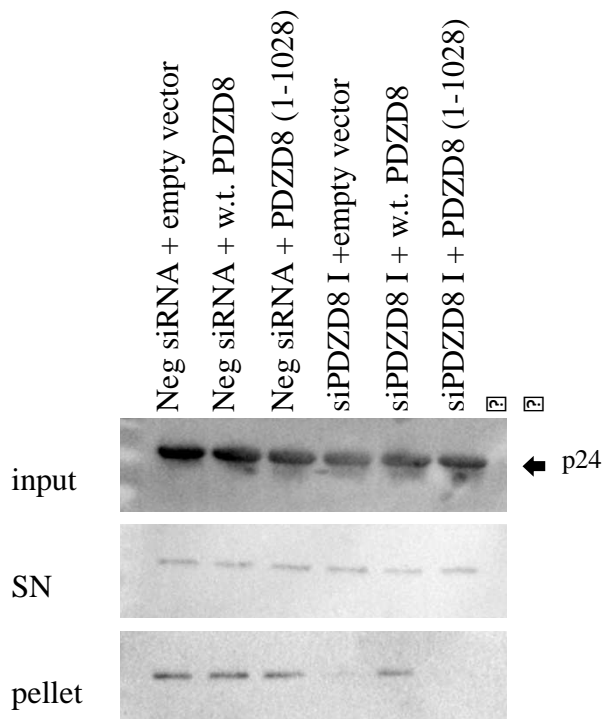


Figure 3.3 Contribution of PDZD8 to stabilization of the HIV-1 capsid in infected cells. HeLa cells were transfected with the negative-control (neg) siRNA or the PDZD8-targeting siPDZD8 I as well as the pIRES vector expressing FLAG-tagged wild-type (w.t.) PDZD8 or the C-terminally truncated PDZD8(1-1028), or an empty vector. The transfected HeLa cells were then incubated with VSV G-pseudotyped HIV-1. The fate-of-capsid assay was used to compare the rate of capsid uncoating. At 16 hours after infection, cells were lysed in 250 μ l hypotonic lysis buffer by a 1-min treatment with a Kontes pestle. Fifty μ l of each sample was saved as an input fraction. The remaining sample was loaded onto a 7-ml 50% sucrose cushion and spun at 125,000 \times g for 2 hours to separate soluble capsid protein in the supernatant (SN) from the particulate capsid in the pellet. The supernatant and resuspended pellet fractions were analyzed by SDS-PAGE and western blotting using antibodies against p24. This experiment was conducted three times and the results from a typical experiment are shown.

Contribution of PDZD8 to retrovirus infectivity.

Previous work has demonstrated a strong correlation between increased kinetics of HIV-1 core uncoating and decreases in viral infectivity (10, 41). Knockdown of PDZD8 by a specific siRNA resulted in an approximately 10-fold decrease in HIV-1 infectivity in HeLa cells (Figure 3.4A). Transient transfection of an siRNA-resistant vector expressing wild-type PDZD8 restored infectivity to a level comparable to that seen in cells transfected with the negative-control

siRNA. By contrast, expression of the PDZD8(1-1028) mutant with a C-terminal truncation did not rescue HIV-1 infectivity in HeLa cells in which PDZD8 expression was knocked down. These results suggest that wild-type PDZD8 contributes to HIV-1 infectivity, consistent with an earlier report (17).

To determine the effect of PDZD8 knockdown on HIV-1 reverse transcription, we performed quantitative PCR to detect levels of strong-stop cDNA, an early reverse transcription product, at 4 hours following infection. In PDZD8 knockdown cells, the level of HIV-1 strong-stop cDNA products was decreased (Figure 3.4B). The production of strong-stop cDNA could be rescued by wild-type PDZD8 but not PDZD8(1-1028). Of note, in the negative-control siRNA-treated cells, the level of HIV-1 strong-stop cDNA was increased in the cells transfected with the plasmid expressing wild-type PDZD8, but not in cells expressing the PDZD8(1-1028) mutant. Although HIV-1 infectivity was not significantly increased in the cells overexpressing the wild-type PDZD8 (see above), this observation suggests that, for some aspects of early HIV-1 infection, endogenous PDZD8 levels may be rate-limiting. The results support a model in which PDZD8 interaction with the HIV-1 capsid contributes to core stability and virus infectivity. Our findings also add to mounting evidence that premature capsid uncoating disrupts early HIV-1 reverse transcription (2, 19, 23, 41).

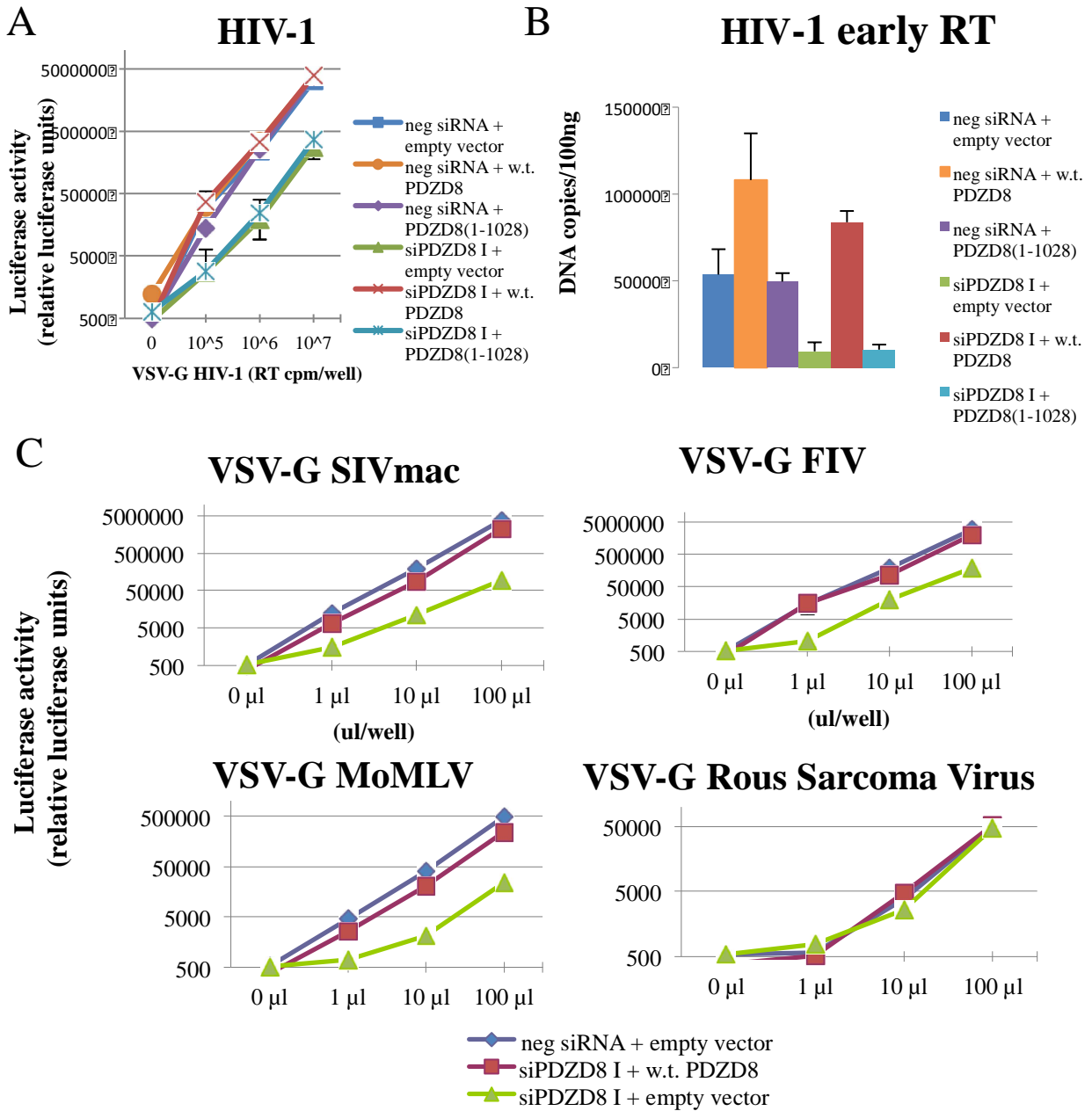


Figure 3.4 PDZD8 exerts positive effects on HIV-1 reverse transcription and infection of several retroviruses. (A) Knockdown of PDZD8 reduces HIV-1 infectivity, and infectivity is rescued by exogenous expression of siRNA-resistant PDZD8. HeLa cells were transfected with the pIRES2 vector expressing FLAG-tagged wild-type (w.t.) PDZD8 or a C-terminal truncation mutant, PDZD8(1-1028), or an empty vector. These cells were also transfected with either a PDZD8-targeting siRNA, siPDZD8 I, or a negative-control (neg) siRNA. Forty-eight hours

Figure 3.4 (continued) following siRNA transfection, cells were incubated with the indicated amount of VSV G-pseudotyped luciferase-expressing HIV-1 virus. Forty-eight hours after infection, cells were lysed and luciferase activity assayed. The means and standard deviations of three independent experiments are reported. (B) Knockdown of PDZD8 inhibits HIV-1 reverse transcription. Cells transfected with PDZD8-targeting siRNA or control siRNA and siRNA-resistant PDZD8-expressing vectors were infected by VSV G-pseudotyped HIV-1, as described in (A) above. The cells were assayed by qPCR to determine the levels of strong-stop cDNA. The strong-stop cDNA levels for the heat-inactivated virus control were less than 5000 DNA copies/ml (data not shown). The means and standard deviations of three independent experiments are reported. (C) HeLa cells transfected as described in (A) were incubated with the indicated concentrations of VSV G-pseudotyped luciferase-expressing simian immunodeficiency virus (SIVmac), feline immunodeficiency virus (FIV), Moloney murine leukemia virus (Mo-MLV) and Rous sarcoma virus for 4 hr. Forty-eight hours following infection, luciferase activity was assayed. The means and standard deviations of three independent experiments are reported.

We tested the effect of PDZD8 knockdown and siRNA-resistant addback of exogenous PDZD8 on the infectivity of a panel of additional VSV G-pseudotyped luciferase-reporter retroviruses (Figure 3.4C). PDZD8 knockdown decreased the infectivity of the pseudotyped SIVmac, Mo-MLV and FIV. However, PDZD8 knockdown had no apparent effect on the infectivity of the pseudotyped Rous sarcoma virus vector. Apparently, PDZD8 contributes to the infectivity of a number of mammalian retroviruses.

Functional contribution of the PDZD8 carboxyl terminus.

Although the structure of PDZD8 is unknown, putative domains in the protein have been identified by sequence similarity to established structures (Figure 3.5A). PDZD8 was originally identified as a potential HIV-1 Gag-binding partner by a yeast two-hybrid screen. A fragment of PDZD8 comprised of amino acid residues 932-1110 bound a bait fragment of HIV-1 Gag (17). This PDZD8 fragment contains a putative coiled-coil domain (amino acid residues 1028-1063) as well as an additional region (residues 1063-1106) predicted to have significant helical character. To investigate the role of specific carboxy-terminal regions of PDZD8 in the activity of this protein, we created a series of FLAG-tagged PDZD8 mutants with carboxy-terminal

truncations (PDZD8(1-1028), PDZD8(1-1063) and PDZD8(1-1106)). HeLa cells transiently transfected with expression vectors encoding wild-type PDZD8 and these truncation mutants were used in infection assays and as a source of lysate for *in vitro* CA-NC stabilization assays (Figure 3.5, B and C). PDZD8 residues carboxy-terminal to residues 1063, including the putative alpha-helical region, were dispensable for rescue of HIV-1 infectivity and CA-NC stabilization. Deletion of the putative coiled-coil motif (residues 1028-1063) eliminated the ability of exogenous PDZD8 to rescue HIV-1 infectivity in HeLa cells in which the expression of endogenous PDZD8 was knocked down. The PDZD8(1-1028) mutant likewise did not stabilize HIV-1 CA-NC complexes *in vitro*. These results support the importance of the coiled-coil to PDZD8 function as an HIV-1 cofactor.

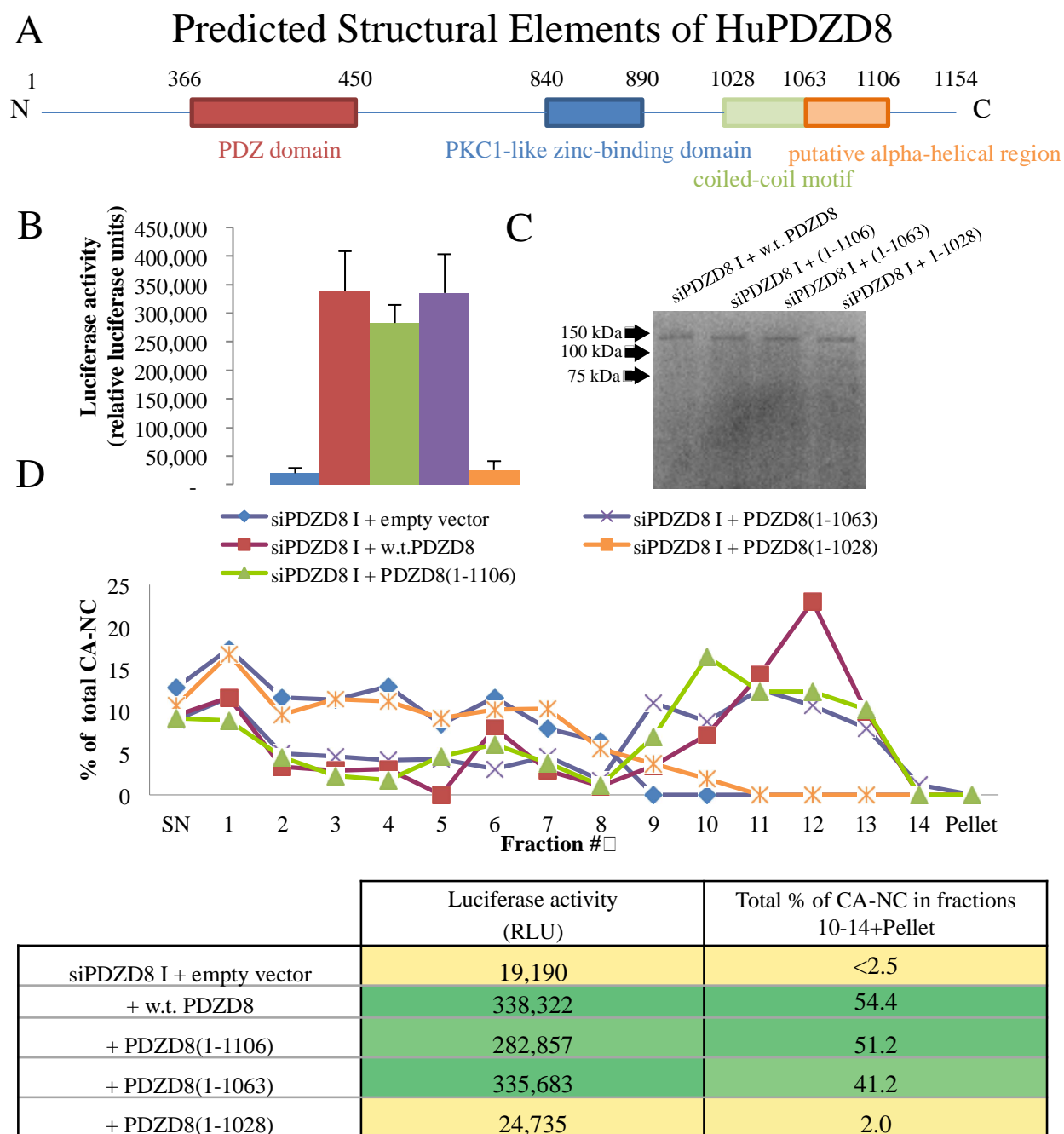


Figure 3.5 Role of C-terminal sequences in PDZD8 function as an HIV-1 cofactor. (A) A schematic of the predicted structural elements of the human PDZD8 protein, with the amino acid residues noted. The domains in this figure are not drawn to scale. (B) HeLa cells transfected with the PDZD8-targeting siRNA siPDZD8 I were also transfected with pIRES vectors expressing N-terminally FLAG-tagged wild-type (w.t.) PDZD8, PDZD8(1-1106), PDZD8(1-1063) and PDZD8(1-1028), or an empty pIRES vector. Cells were incubated with 1×10^6 cpm RT of VSV G-pseudotyped HIV-1 expressing luciferase. Luciferase activity was assessed at 48 hours after infection. The means and standard deviations of three independent experiments are

Figure 3.5 (continued) reported. (C) Western blot of lysates from cells expressing the indicated FLAG-tagged proteins. (D) The HIV-1 CA-NC stabilization assay was performed with lysates from cells transfected as described in (B). The CA-NC content of individual fractions was measured by ELISA and normalized to the total amount of CA-NC in the sample. This experiment was conducted three times and the results from a typical experiment are shown. (E) The chart compares the fraction of total CA-NC located in Fractions 10-14 plus the pellet in the HIV-1 CA-NC stabilization assay shown in (D) with the luciferase activity from the assay described in (B). Cell shading is a two-color gradient calibrated to the minimum and maximum values in each column by Microsoft Excel.

Role of the PDZ and PKC1-like domains in PDZD8 function.

Sequence analysis of PDZD8 indicates the presence of two additional conserved domains, a PDZ domain and a PKC1-like zinc-binding domain (Figure 3.5A). To determine the importance of these regions to PDZD8 function, we progressively deleted N-terminal portions of the protein. However, we found that steady-state levels of PDZD8 N-terminal truncation mutants (both with and without N-terminal FLAG tags) were very low when transiently expressed. N-terminal fusion with GFP substantially increased expression to levels comparable to that of the full-length PDZD8 protein. Therefore, we constructed a series of fusion proteins in which an N-terminal FLAG-tagged GFP molecule is fused with either full-length PDZD8 or N-terminally truncated PDZD8. Loss of the PDZD8 sequences N-terminal to the PDZ domain did not affect PDZD8 rescue of HIV-1 infection or *in vitro* stabilization of HIV-1 CA/NC complexes (See GFP-PDZD8(366-1154) in Figure 3.6). Further N-terminal truncation, which removed the PDZ domain (GFP-PDZD8(450-1154)), resulted in a loss of both CA-NC-stabilizing activity and the ability to rescue HIV-1 infection (Figure 3.6).

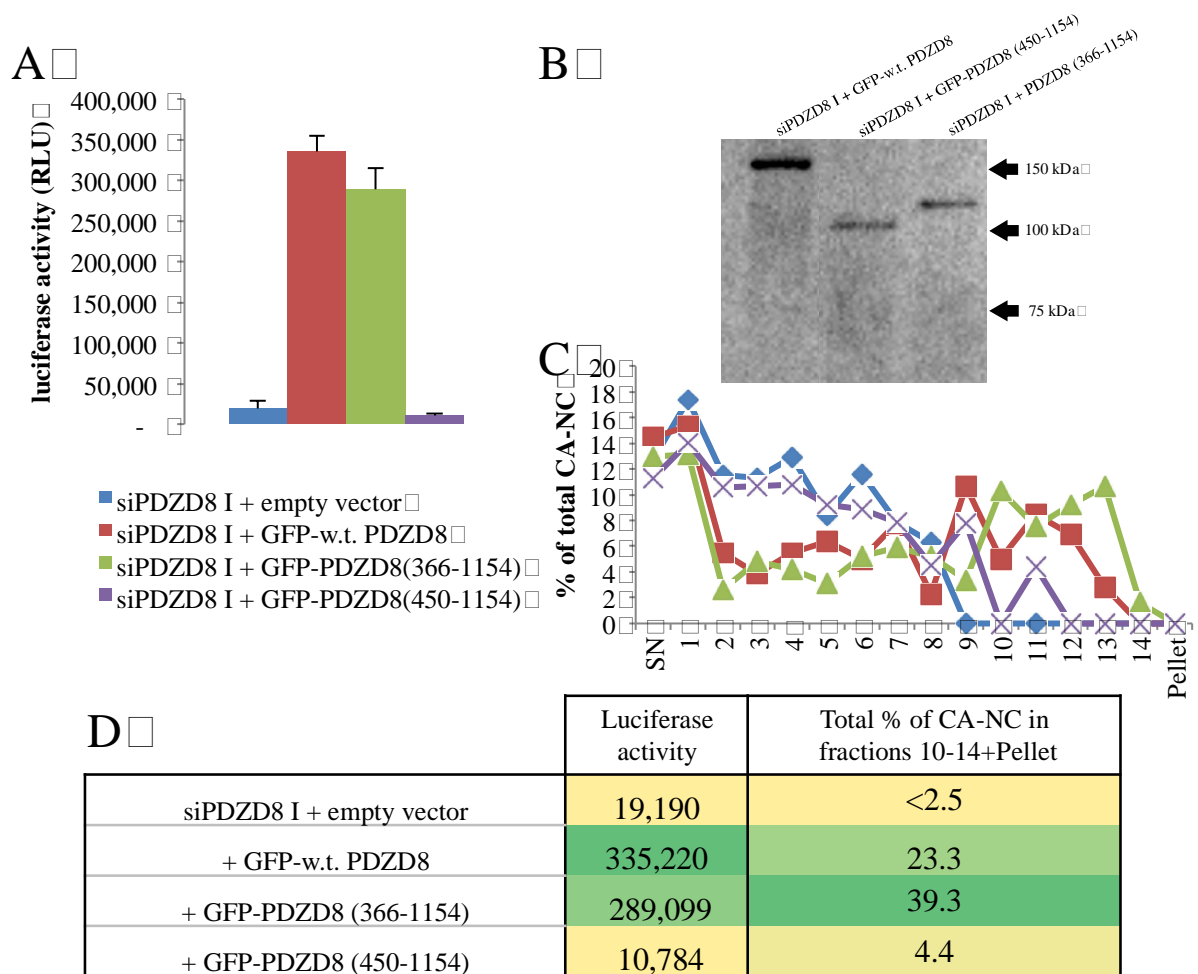


Figure 3.6 Role of the PDZ domain in PDZD8 function as an HIV-1 cofactor. (A) HeLa cells were transfected with the PDZD8-targeting siRNA siPDZD8 I and either the empty pIRES vector or pIRES vectors expressing N-terminally FLAG-tagged GFP-fused wild-type (w.t.) PDZD8, PDZD8(366-1154), and PDZD8(450-1154). Cells were incubated with 1×10^6 cpm RT of VSV G-pseudotyped HIV-1 expressing luciferase, and luciferase activity was assessed at 48 hours following infection. (B) Western blot of lysates from cells expressing the indicated FLAG-tagged proteins. (C) The HIV-1 CA-NC stabilization assay was performed with lysates from cells transfected as described in (A). The CA-NC content of individual fractions was measured by ELISA and normalized to the total amount of CA-NC in the sample. This experiment was conducted three times and the results from a typical experiment are shown. (D) The chart compares the fraction of total CA-NC located in Fractions 10-14 plus the pellet in the HIV-1 CA-NC stabilization assay shown in (B) with the luciferase activity from the assay described in (A). Cell shading is a two-color gradient calibrated to the minimum and maximum values in each column by Microsoft Excel.

PDZ domains are common protein-protein interaction modules with a well-defined binding motif that most commonly binds the C-terminal peptides of target proteins (36). There

are currently no known binding partners of the PDZD8 PDZ domain. To test the hypothesis that the protein-binding function of the PDZ domain is required for the capsid stabilization function of PDZD8, we used site-directed mutagenesis to alter the PDZD8 residues that are putatively located on both sides of the peptide-binding groove of the PDZ domain (9). These PDZD8 mutants rescued HIV-1 infectivity and *in vitro* CA-NC stabilization following PDZD8 knockdown with efficiencies comparable to those seen for wild-type PDZD8 (Figure 3.7). Thus, the putative peptide-binding groove of PDZD8 can be substantially altered without affecting either PDZD8 stabilization of HIV-1 CA-NC capsids *in vitro* or rescue of HIV-1 infectivity.

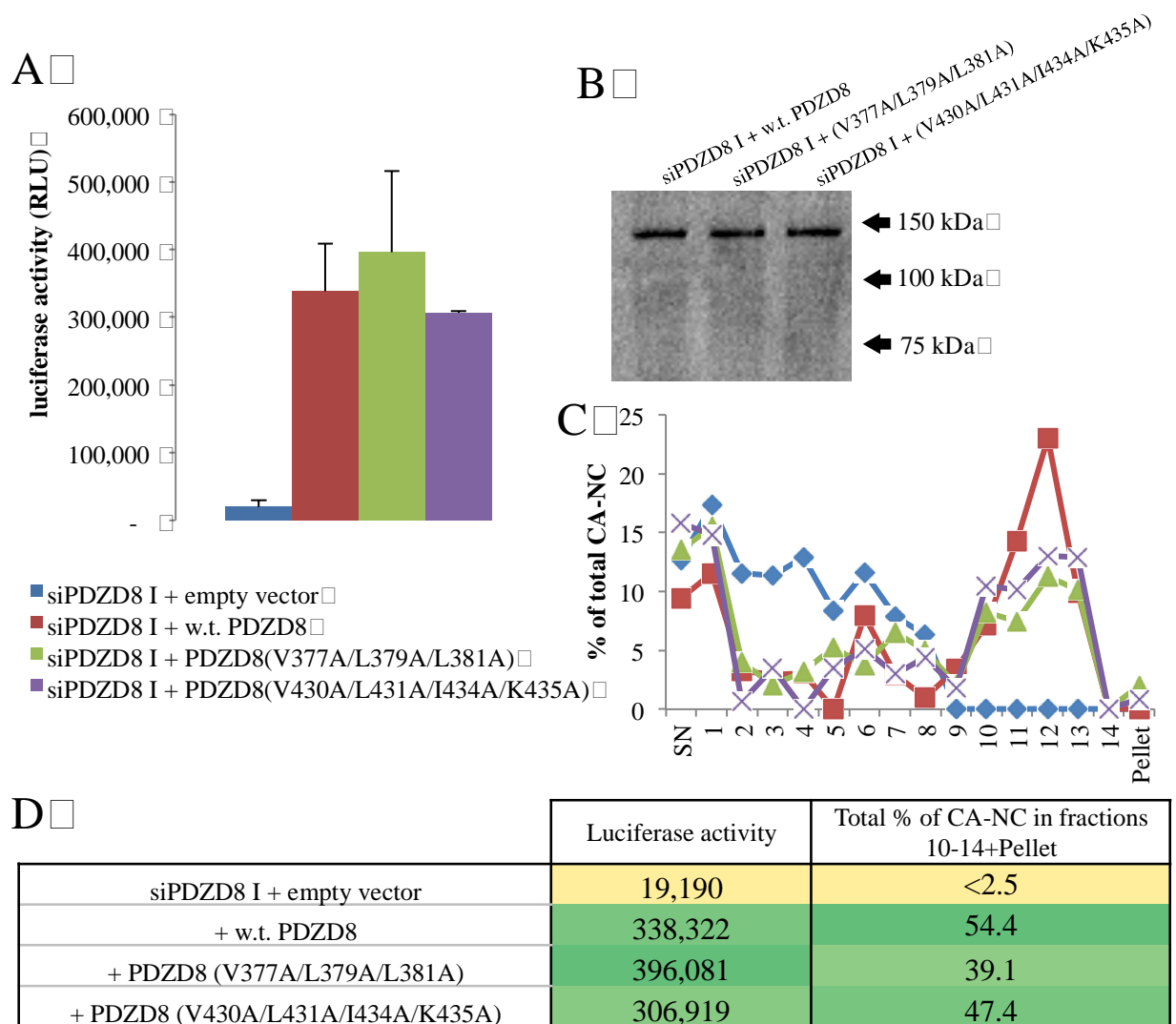


Figure 3.7 Role of the PDZ peptide-binding motif in PDZD8 function as an HIV-1 cofactor. (A) HeLa cells were transfected with the PDZD8-targeting siRNA siPDZD8 I and either the empty pIRES vector or pIRES vectors expressing N-terminally FLAG-tagged wild-type (w.t.) PDZD8, or full-length PDZD8 with alanine substitutions in the PDZ peptide-binding motif (V430A/L431A/I434A/K435A and V377A/L379A/L381A). Cells were incubated with 1×10^6 cpm RT of VSV G-pseudotyped HIV-1 expressing luciferase, and luciferase activity was assessed at 48 hours following infection. (B) Western blot of lysates from cells expressing the indicated FLAG-tagged proteins. (C) The HIV-1 CA-NC stabilization assay was performed with lysates from cells transfected as described in (A). The CA-NC content of individual fractions was measured by ELISA and normalized to the total amount of CA-NC in the sample. This experiment was conducted three times and the results from a typical experiment are shown. (D) The chart compares the fraction of total CA-NC located in Fractions 10-14 plus the pellet in the HIV-1 CA-NC stabilization assay shown in (B) with the luciferase activity from the assay described in (A). Cell shading is a two-color gradient calibrated to the minimum and maximum values in each column by Microsoft Excel.

Additionally, we constructed a FLAG-tagged PDZD8 mutant with an internal deletion of the PKC domain (Δ 840-890). The PDZD8(Δ 840-890) mutant, which lacks the PKC1-like domain, rescued HIV-1 infectivity in PDZD8 knockdown cells and CA-NC-stabilizing activity in PDZD8-depleted HeLa cell lysates (Figure 3.8). Thus, the PKC1 domain is not apparently required for PDZD8 function with respect to HIV-1 infection. Finally, we created a construct removing all residues between the PDZ domain and the linker region preceding the coiled-coil motif, PDZD8(Δ 451-1016). Deletion of either PDZD8's N-terminal region, which contains the PDZ domain, or its C-terminal region, which contains the coiled-coil motif, eliminates CA-NC stabilization. We hypothesized that these two domains might be sufficient for stabilization and infectivity enhancement. This construct did not restore infectivity of stabilization in PDZD8 depleted cells and lysate, suggesting that either some as yet unidentified region within the deleted segment is important to stabilization function or that a deletion of this scale prevents the proper folding or arrangement of the PDZ and coiled-coil domains required for stabilization (Figure 3.8).

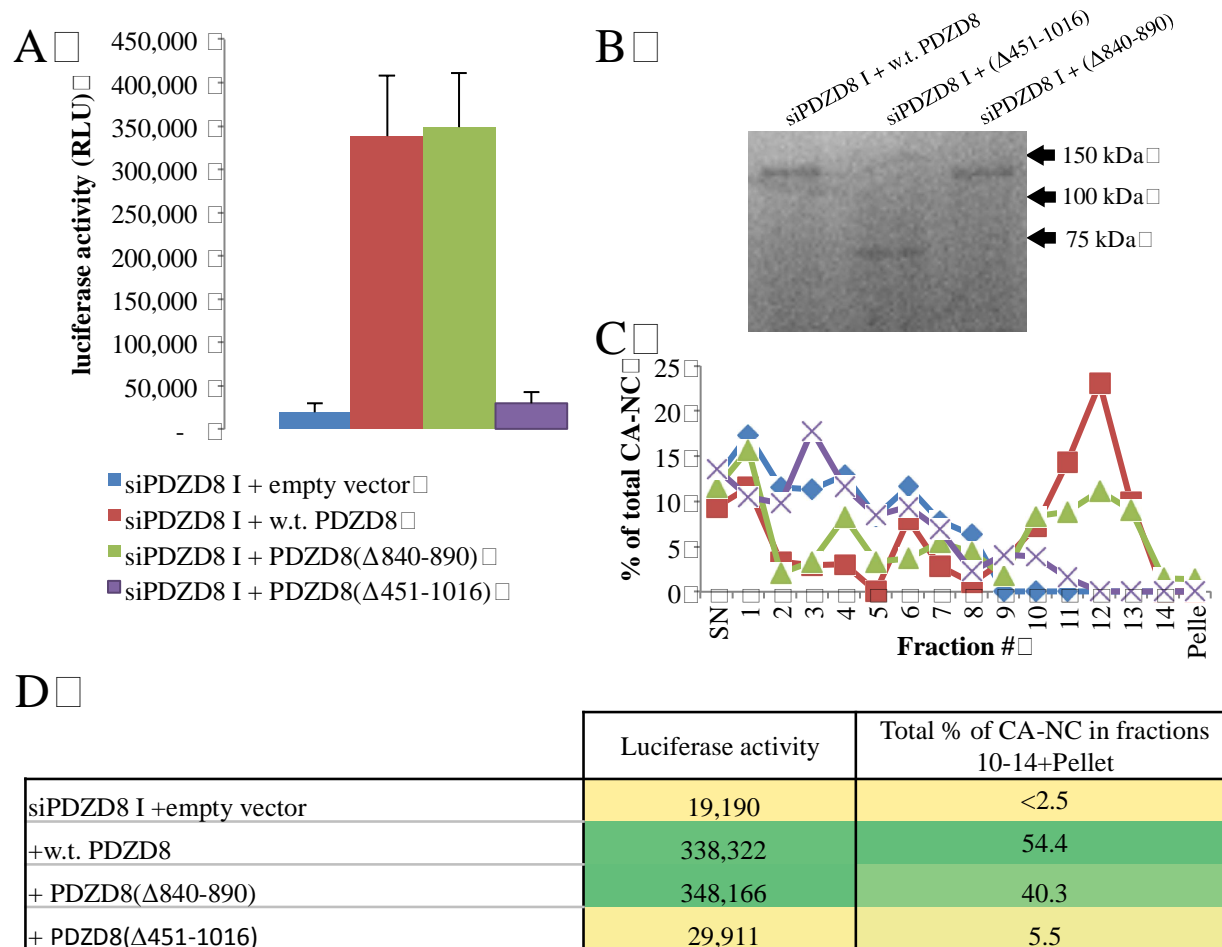


Figure 3.8 Role of the PKC1 domain in PDZD8 function as an HIV-1 cofactor. (A) HeLa cells were transfected with the PDZD8-targeting siRNA siPDZD8 I and either the empty pIRES vector or pIRES vectors expressing N-terminally FLAG-tagged wild-type (w.t.) PDZD8, PDZD8(Δ840-890) with the PKC1-like domain deleted, a PDZD8 deleting all residues between the N-terminal PDZ domain and C-terminal CC motif required for stabilization PDZD8(Δ451-1016). Cells were incubated with 1×10^6 cpm RT of VSV G-pseudotyped HIV-1 expressing luciferase, and luciferase activity was assessed at 48 hours following infection. (B) Western blot of lysates from cells expressing the indicated FLAG-tagged proteins. (C) The HIV-1 CA-NC stabilization assay was performed with lysates from cells transfected as described in (A). The CA-NC content of individual fractions was measured by ELISA and normalized to the total amount of CA-NC in the sample. This experiment was conducted three times and the results from a typical experiment are shown. (D) The chart compares the fraction of total CA-NC located in Fractions 10-14 plus the pellet in the HIV-1 CA-NC stabilization assay shown in (B) with the luciferase activity from the assay described in (A). Cell shading is a two-color gradient calibrated to the minimum and maximum values in each column by Microsoft Excel.

PDZD8 regions involved in HIV-1 capsid binding.

To investigate the contribution of PDZD8 domains to the interaction of PDZD8 with the HIV-1 core, we evaluated the ability of PDZD8 mutants to co-sediment with HIV-1 CA-NC complexes. FLAG-tagged PDZD8 variants were expressed transiently in HeLa cells, and cell lysates were incubated with *in vitro* assembled HIV-1 CA-NC complexes for 3 hours. Binding of the PDZD8 variants to the CA-NC complexes was assessed after sedimentation through a 70% sucrose cushion and detection of the FLAG-tagged PDZD8 protein by western blotting. Loss of the PDZ domain, which resulted in undetectable CA-NC-stabilizing ability (see above), did not affect the interaction with HIV-1 CA-NC complexes (See GFP-PDZD8(450-1154) in Figure 3.9). The putative coiled-coil motif of PDZD8 was both necessary and sufficient for HIV-1 CA-NC binding (See GFP-PDZD8(1017-1063) in Figure 3.9). These results are consistent with a previous report that deletion of a C-terminal fragment of PDZD8 (residues 1028-1154) abrogates the co-immunoprecipitation of PDZD8 by HIV-1 Gag/Pol (17). These results also indicate that HIV-1 capsid binding by PDZD8 is necessary but not sufficient for capsid stabilization and activity as an infection cofactor.

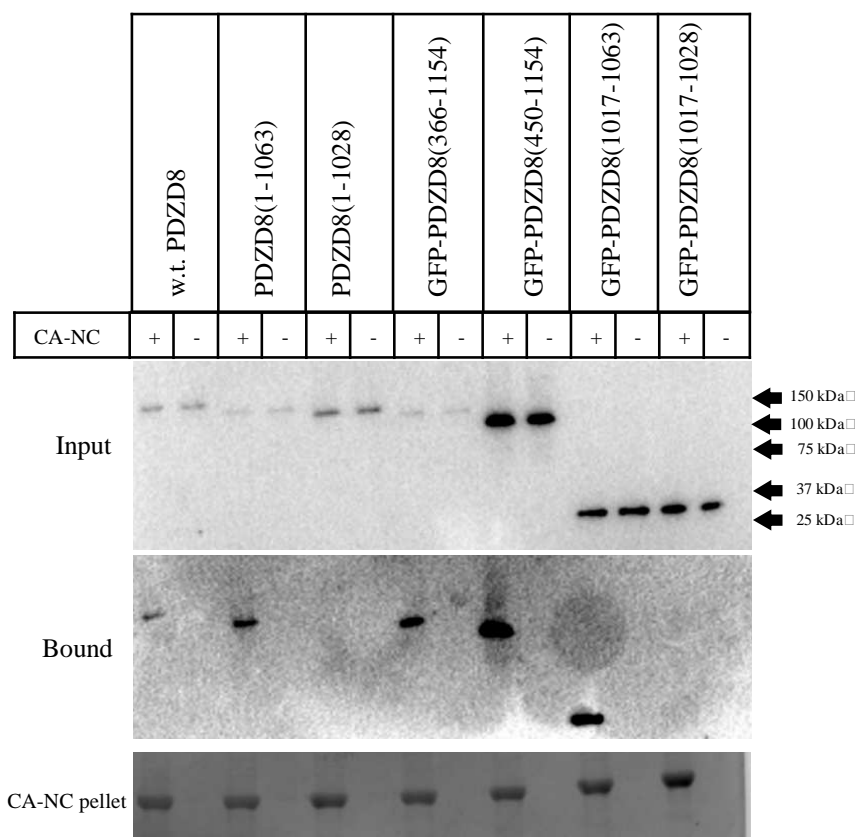


Figure 3.9 The coiled-coil motif of PDZD8 is necessary and sufficient for binding to HIV-1 CA-NC complexes. Lysates derived from HeLa cells transfected with pIRES vectors expressing FLAG-tagged wild-type (w.t.) PDZD8 or the indicated PDZD8 variants were mixed with *in vitro* assembled HIV-1 CA-NC complexes for 2 hours at room temperature. As indicated, some truncations of PDZD8 were fused to an N-terminal GFP protein to enhance expression. Negative-control experiments lacking CA-NC complexes were performed in parallel. After mixing, 50 μ l of the mixture was saved as an input fraction. The remaining mixture was loaded on a 3.5-ml 70% sucrose cushion and spun at 110,000 $\times g$ for 2 hr. The supernatant and sucrose cushion were removed and the pellet fraction was resuspended in 100 μ l 1x SDS sample buffer. Input and pellet fractions were analyzed by SDS-PAGE. The SDS-polyacrylamide gel was analyzed by staining with Coomassie Blue to detect the CA-NC protein and Western blotted with an anti-FLAG antibody to detect bound PDZD8. The experiment was conducted three times, and the results from a typical experiment are shown.

Discussion

Host restriction factors that accelerate the uncoating of retroviral capsids have been extensively studied (30). The identity and mechanism of action of host factors that stabilize

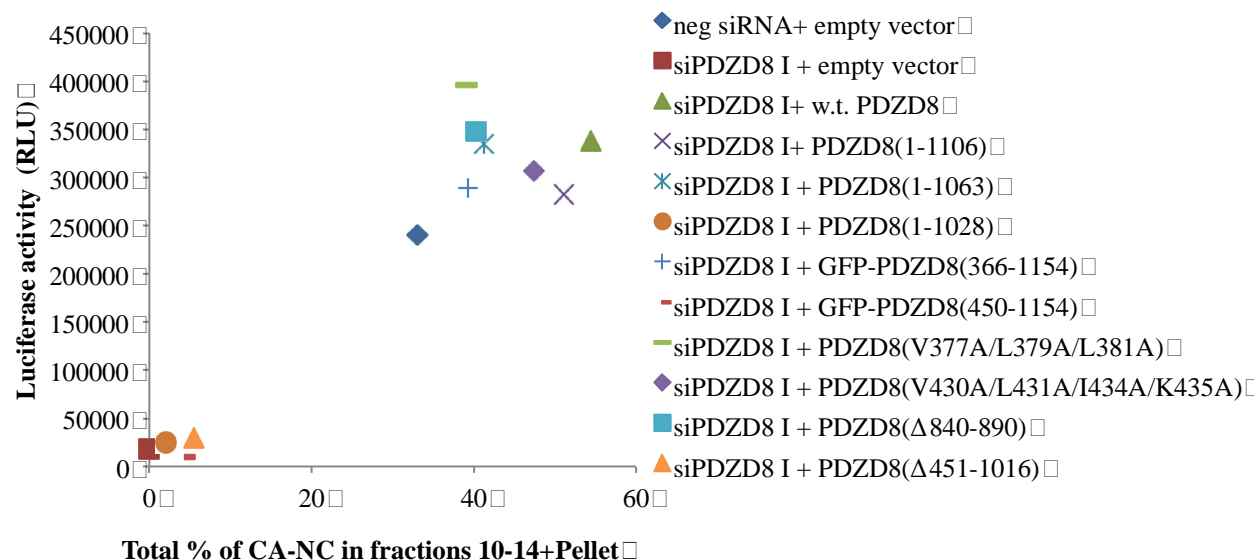
retroviral capsids and act as positive cofactors for infection are less well understood. Here we describe an *in vitro* assay to measure the stabilization of HIV-1 capsid-like assemblies by host cell lysates. We used this assay to identify PDZD8 as a major capsid-stabilizing factor in cell lysates. PDZD8 is a known HIV-1 Gag-interacting factor that promotes viral infection in advance of the completion of reverse transcription (17). By implicating PDZD8 in the stabilization of HIV-1 capsid-like assemblies *in vitro* and HIV-1 viral capsids in infected cells, we provide a mechanism for the observations of Henning et al. (17).

A very limited number of cellular proteins have been implicated in the regulation of retroviral capsid uncoating and only one, cyclophilin A (CypA), is known to enhance lentiviral infection by stabilizing the capsid (27). CypA represents an interesting reference for a consideration of the role of PDZD8 in the retroviral life cycle. Target cell PDZD8, like CypA, is apparently utilized by multiple diverse retroviruses. In the case of CypA, studies of archaic retroviruses indicate that lentiviral CypA-CA interactions likely predate HIV-1 emergence by at least 12 million years, underscoring the important and highly conserved role of this capsid-stabilizing cofactor in infection of some lentiviruses (15). PDZD8 knockdown affected infection by several lentiviruses and a gammaretrovirus, but not Rous sarcoma virus. Thus, although CypA and PDZD8 act as positive cofactors for diverse retroviruses, some retroviruses apparently do not utilize these host cofactors. It is of interest to understand whether the latter retroviruses employ other capsid-stabilizing strategies. The comparison to CypA also invites future work to consider the capsid stabilization effect of PDZD8 in a broad range of species and cellular contexts. Both CypA and PDZD8 are well conserved host vertebrate species. The effect of CypA-CA binding on HIV-1 infectivity is highly dependent on target cell type, potentially due to differing levels of expression of CypA or CypA-mediated modulation of the susceptibility of

HIV-1 to co-expressed viral restriction factors (27, 42). Retroviral phenotypes that result from modulation of PDZD8 levels may be similarly context-dependent. In CHME3 brain microglia and 293A kidney epithelial cells, overexpression of PDZD8 by transfection has been reported to increase HIV-1 infectivity and early reverse transcript production by approximate 10-fold (17). In HeLa cells, we observed only a small enhancement (<2-fold) of HIV-1 infectivity from a similar transfection. This difference is potentially related to differences in endogenous expression of PDZD8 host cell lines. Quantitation of PDZD8 protein levels is difficult due to the current unavailability of suitable antibodies for detection of endogenous PDZD8 protein. Alternatively, these discrepancies in infectivity phenotypes may reflect differences in other critical elements dictating capsid stability and reverse transcription.

We observed an excellent correlation between the PDZD8 mutant phenotypes in the *in vitro* HIV-1 CA-NC stabilization assay and the HIV-1 infectivity assay (Figure 3.10). Deletion of the PDZD8 coiled-coil motif abrogated the stabilizing effect of PDZD8 on CA-NC assemblies *in vitro* and resulted in loss of the ability to support HIV-1 infection. The coiled-coil domain appears to be necessary and sufficient for the interaction of PDZD8 with HIV-1 CA-NC complexes. The coiled-coil domain potentially plays a role in the multimerization of PDZD8, although this has yet to be demonstrated. Of note, multimerization of the capsid-destabilizing factor TRIM5 α enhances binding to the viral capsid (20, 26). One model of restriction proposes that TRIM5 α binding to the capsids of unrelated retroviruses is achieved by recognition of conserved hexameric patterns on the assembled capsid surface by TRIM5 α multimers (14). Future studies should address the natural oligomeric state of PDZD8 and its contribution to recognition of the retroviral capsid.

N-terminal deletion of the first 365 residues of PDZD8 exerted little impact on its function in stabilization of the HIV-1 capsid. However, a further N-terminal deletion that removed the PDZ domain was inactive, even though this mutant still bound the HIV-1 CA-NC complexes. Surprisingly, the peptide-binding motif of PDZD8, a common target for pathogenic viruses in other PDZ-containing proteins (21), does not appear to be required for capsid interaction, stabilization, or enhancement of infection. Additional studies will be required to determine the role of the PDZ domain in the function of PDZD8 as an HIV-1 cofactor.



	Luciferase activity	Total % of CA-NC in fractions 10-14+Pellet
neg siRNA+ empty vector	241,304	33
siPDZD8 I+ empty vector	19,190	<2.5
siPDZD8 I+ w.t. PDZD8	338,322	54.4
siPDZD8 I+ PDZD8(1-1106)	282,857	51.2
siPDZD8 I+ PDZD8(1-1063)	335,683	41.2
siPDZD8 I+ PDZD8(1-1028)	24,735	<2.5
siPDZD8 I+ GFP-PDZD8(366-1154)	289,099	39.3
siPDZD8 I+ GFP-PDZD8(450-1154)	10,784	4.4
siPDZD8 I+ PDZD8(V377A/L379A/L381A)	396,081	39.1
siPDZD8 I+ PDZD8(V430A/L431A/I434A/K435A)	306,919	47.4
siPDZD8 I+ PDZD8(Δ 840-890)	348,166	40.3
siPDZD8 I+ PDZD8(Δ 451-1016)	29,911	5.5

Pearson's correlation coefficient	0.901
p-value	<0.001

Figure 3.10 Comparison of HIV-1 infectivity enhancement and CA-NC *in vitro* stabilization by PDZD8 constructs. The data are derived from the experiments described in Figures 3.5-3.8 by methods described in those figure legends. Cell shading is a two-color gradient calibrated to the minimum and maximum values in each column by Microsoft Excel.

The C-terminal region of PDZD8, which includes the coiled-coil motif, has also been implicated in the interaction of PDZD8 with the cytoskeletal regulation protein moesin (18). Moesin overexpression has pleiotropic effects on HIV-1 infections, including a negative post-

entry, pre-reverse transcription effect coincident with the timing of capsid uncoating (4, 22, 32). One explanation for moesin restriction of HIV-1 infection is that infectivity is diminished due to inhibition of PDZD8-capsid interaction as a result of the over-expressed moesin competing for access to PDZD8's C-terminal region.

Finally, the PKC1-like domain of PDZD8 appears to be completely dispensable for HIV-1 CA-NC-stabilizing activity and for the ability to act as a cofactor for HIV-1 infection.

Following retrovirus entry into the host cell, the uncoating of the viral capsid may be determined by the binding of stabilizing host factors including PDZD8 and CypA in competition with the binding of cellular restriction factors like TRIM5 α and TRIMCyp. Additional inquiry into this critical stage of the viral life cycle may suggest interventional approaches, such as interruption of the PDZD8-capsid interaction, that could tip the balance towards host cell resistance

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Chapter Four

Discussions

4.1.0 Discussion and impact

4.1.1 Development of an *in vitro* assay of HIV-1 core stability

During the past decade, a number of cellular proteins have been identified as regulating factors of retroviral capsid uncoating, a process of critical importance to viral infectivity (8, 11, 30). However, the study of uncoating has been limited by the absence of suitable *in vitro* assays. I pursued the development of *in vitro* uncoating assays as a complement to the more established protocols for assaying uncoating *in vivo*. Of particular interest was the development of a protocol that would be useful for the identification of additional core-stabilizing or core-destabilizing factors. Current protocols for assaying uncoating *in vivo* are low-throughput and technically difficult, making screening for novel capsid-stabilizing factors challenging (3, 17, 32). Additionally, because these assays require viable cells, fractionation of cellular components is impractical. This limits investigators' abilities to isolate individual capsid-binding cofactors, which is problematic both for the identification of novel capsid-stabilizing factors and for the mechanistic studies of previously identified factors.

Though previously developed *in vitro* assays of capsid stability have been described, these assays are largely incapable of measuring the impact of cellular proteins on core uncoating. Of those few analyses purported to measure cofactor effects on HIV-1 core stability have, all but one (29) have measured only enhanced capsid destabilization and all in the presence of high concentrations of known capsid-binding proteins (7, 14, 28, 34). For this reason, it is unclear whether the cofactor-dependent capsid disassembly observed in these assays is analogous to the accelerated capsid uncoating that these

factors induce *in vivo*, or whether this disassembly is an epiphenomenon due, for example, to steric hindrance by abundant CA-binding factors.

Here, I have presented an *in vitro* assay of HIV-1 core stability that makes use of purified CA-NC complexes as a capsid analog. Stabilization of CA-NC complexes *in vitro* by PDZD8 and related constructs correlates well with infectivity *in vivo*. This is the first assay to measure the effect of cellular factors on retroviral stability that does not require purification or transient overexpression of the cellular factor. Furthermore, the assay is higher-throughput and its results more easily quantitated than comparable *in vivo* and *in vitro* protocols, including the fate-of-capsid assay and EM analysis of CA-NC disassembly (7, 32). These properties give this novel assay a high potential for usefulness in future experimental investigation of retroviral core uncoating and the effect of cellular factors on this process.

4.1.2 Existence of a novel stabilizing factor

The first notable experimental finding generated with the CA-NC tube stabilizing assay was the discovery of CA-NC-stabilizing properties in human cell lysates. Subsequent work demonstrated that the effect was likely the result of a protein component or components of the lysate, but that CA-NC stabilization is not a non-specific property of all proteins. Stabilization potential is present in lysate from a wide range of cell types including human, mouse, dog and chicken suggesting that the stabilizing factor is well conserved.

This finding is an interesting one in the context of the ongoing investigation of retroviral capsid stability. Early *in vitro* analysis has demonstrated that purified retroviral

capsids are very unstable in physiological buffers (11). Based in part on the rapid uncoating of purified cores *in vitro*, retroviral uncoating has been hypothesized to occur shortly after viral membrane fusion (2). However, this model is difficult to reconcile with more recent *in vivo* experimental evidence suggesting that core stability in the cytoplasm is critical to retroviral infectivity. Accelerating core uncoating by a small molecule or restriction factor has a significant negative impact on retroviral infectivity (26, 30, 31). Analysis by the *in vivo* fate-of-capsid assay indicates that retroviral cores are relatively stable following entry into the cytoplasm, with a half-life of 8-10 hours (B. Bollman, unpublished). The discovery of a capsid-stabilizing component suggests an explanation for the divergence between this *in vitro* and *in vivo* data.

At the time of this finding, the only identified HIV-1-stabilizing cellular cofactor was CypA. Fractionation of human cell lysates demonstrated that the significant CA-NC-stabilizing effect by cytoplasmic lysate was not dependent upon the presence of CypA (23). This suggested that lysate stabilization of CA-NC reflected the existence of a previously unidentified core-stabilizing factor. Subsequent to this research, the cellular factor CPSF6 was also shown to stabilize HIV-1 cores (10, 19). CPSF6, however, is not present in large amounts in cytoplasmic lysates and is not the primary stabilizing lysate component identified in these assays.

4.1.3 PDZD8 stabilizes the HIV-1 core

Here I present evidence that human PDZD8 stabilizes HIV-1 CA-NC complexes *in vitro* and the viral core during infection. Prior to this study, PDZD8 had been identified as a positive cofactor of HIV-1 infection, though the role of the factor in the

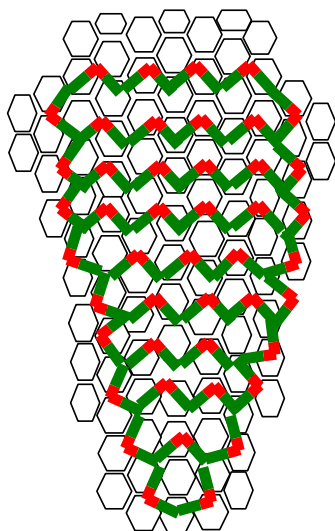
viral lifecycle was unknown (15). PDZD8 is just the second positive cofactor of HIV-1 infection determined to act by promoting stability of the viral capsid. Knockdown of PDZD8 by siRNA increases the uncoating rate of the HIV-1 capsid and decreases viral infectivity. This PDZD8-dependent effect on viral infectivity was also observed for infection by SIVmac, FIV, and MoMLV, but not by Rous sarcoma virus (RSV). This stabilization appeared to require both the putative PDZ domain and coiled-coil motif of PDZD8. Although the specific role of the PDZ domain remains unknown, the coiled-coil motif has been shown to be necessary for the recognition of HIV-1 CA protein. Continued investigation into the specific roles of individual domains of PDZD8 in capsid stabilization will be a critical component of future work.

The identification of a novel retroviral core-stabilizing factor is an important development in the study of HIV-1 uncoating. The discovery of previously identified capsid-stabilizing and destabilizing factors were catalysts in the development of new models of viral uncoating and contributed to the development of novel assays of capsid stability *in vivo* (1, 17, 32, 33). PDZD8 is of particular interest because its action can be observed *in vivo* and in the *in vitro* assay described above. I believe the availability of both *in vitro* and *in vivo* assays will be advantageous in future mechanistic studies of PDZD8 stabilization that may, in turn, provide broader insights into the poorly understood process of retroviral uncoating. Additionally, as PDZD8 is necessary for efficient retroviral infection of some cell lines, investigation into inhibitors of this process may prove to be valuable in the ongoing pursuit of HIV-1 antivirals.

4.2.0 Potential mechanisms of capsid stabilization by PDZD8

The specific mechanism by which PDZD8 contributes to the stabilization of the retroviral capsid is currently unknown. This aspect of PDZD8 is quite similar to the specific stabilization and destabilization mechanisms of all other previously identified cofactors and restriction factors targeting retroviral uncoating, all of which are also poorly understood. Investigation into regulation of capsid uncoating by factors such as TRIM5 α and CypA has been hampered by the challenges of isolating stable capsids for *in vitro* studies and the isolation of cellular factors of interest in *in vivo* studies. The development of the *in vitro* CA-NC stability assay may be a valuable tool in overcoming these technical obstacles and driving forward mechanistic studies of these factors as well as of PDZD8. Based on the data presented in chapters 2 and 3, we would propose that investigation of PDZD8 stabilization focus first on two models –*CA linkage by multimeric PDZD8* and *cofactor recruitment*.

CA linkage by multimeric PDZD8 □
(Section 4.2.1) □



Co-factor recruitment □
(Section 4.2.2) □

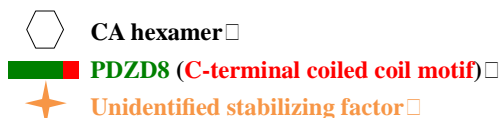
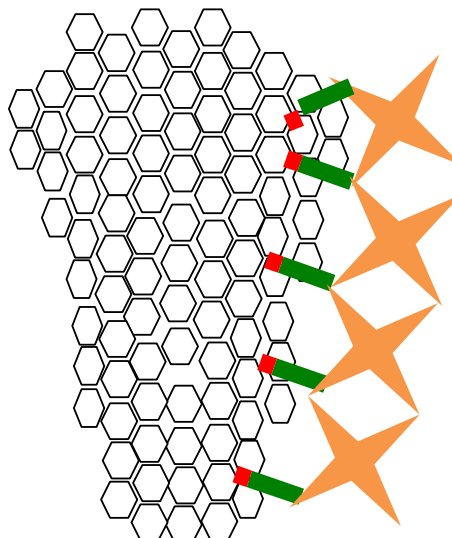


Figure 4.1 Potential mechanisms of capsid stabilization by PDZD8.

4.2.1 CA linkage by multimeric PDZD8

In one hypothetical model of capsid stabilization, a multimeric array of PDZD8 acts to directly stabilize the viral capsid by physically binding together the CA molecules, maintaining high local CA concentration and preventing disassociation and disassembly. It has been theorized that inherently unstable capsids may disassemble following viral membrane fusion due to the release of the concentrated CA protein from the virion into the cytoplasm (2). In the constrained, CA-rich environment of the virion, any disassociation of component CA from the viral capsid might be quickly reversed by reincorporation of that CA into the capsid or the replacement of that CA by free CA molecules. However, following viral core entry into the cytoplasm, CA-CA

disassociation is less likely to be reversed, as capsid components can more easily disperse, leading to the eventual disassembly of the intact capsid structure.

A multimeric lattice of PDZD8 might function to slow this process by minimizing CA disassociation. Our research indicates that the coiled-coil motif of PDZD8 is necessary and sufficient to bind CA protein. A multimeric array of such proteins might be capable of binding multiple CA molecules incorporated into a single capsid structure. CA molecules linked by such an array would experience less dispersion following loss of CA-CA interaction, increasing the local concentration of CA protein and allowing an opportunity for the restoration of the intact lattice, increasing the stability of the whole capsid structure.

This model bears some similarities to a proposed mechanism of TRIM5 α destabilization. Like PDZD8, TRIM5 α has a coiled-coil motif. With TRIM5 α that motif is required for higher-order self-association, and that higher-order self-association is required for retroviral restriction (21, 22). Imaging of a TRIM5 α analog has suggested that TRIM5 α may form a multimeric structure with hexameric symmetry (13). It has been hypothesized that a rigid TRIM5 α lattice might bind multiple CA molecules within a single capsid, potentially exerting force that wrenches bound CA molecules from alignment within the capsid. Binding of purified TRIM5 α proteins has been reported to decrease the size of CA-NC tubes *in vitro*, by destabilizing inter-hexameric interactions (34). I hypothesize that a PDZD8 lattice that either more fully matches the curvature of the retroviral capsid or that has greater flexibility between CA-binding sites might induce an opposite effect, enhancing capsid or CA-NC complex stability.

Some early evidence is consistent with the hypothesis that PDZD8 self-associates into larger multimers. Sequence analysis of PDZD8's coiled-coil motif suggests that this element may form homo-dimers. Chemical cross-linking of a purified, bacterially expressed fragment of the PDZD8 C-terminal region that includes the coil, PDZD8(1017-1154) results in complexes of sizes consistent with dimers and other higher order complexes (Figure 4.1). In preliminary experiments, chemical cross-linking of full-length tagged PDZD8 protein also results in larger complexes, though it is unclear if those larger complexes are the result of self-association. Surprisingly, cross-linking of tagged PDZD8 constructs lacking the coiled-coil motif, PDZD8(1-1028), also form these larger complexes. It is possible that, as is the case for TRIM5 α , multiple domains of the protein contribute to multimerization. However, I cannot yet rule out the possibility that those large complexes are not due to self-association but are a result of PDZD8 forming complexes with other factors. Interestingly, lysate that passes through 300-kDa NWCO filters, is capable of CA-NC tube stabilization (Figure 2.7A). This filter pore size would not be anticipated to permit PDZD8 multimers larger than dimers to pass through. This suggests that if multimerization is required for stabilization, then either dimerization is sufficient for this effect or additional higher-order multimerization occurs post-filtration. Data from size-exclusion chromatography would be consistent with the second hypothesis. Lysate fractions predicted to contain proteins equivalent in size to PDZD8 monomers also demonstrate stabilization capability (Figure 2.7B). It is possible that multivalent assembled capsid may stimulate PDZD8 higher-order self-association by bringing multiple CA-bound PDZD8 molecules into close proximity. Future work will be required to confirm that PDZD8 forms higher-order self-association complexes,

identify those regions of PDZD8 that contribute to higher-order self-association, and determine if that self-association is a requirement for retroviral core stabilization.

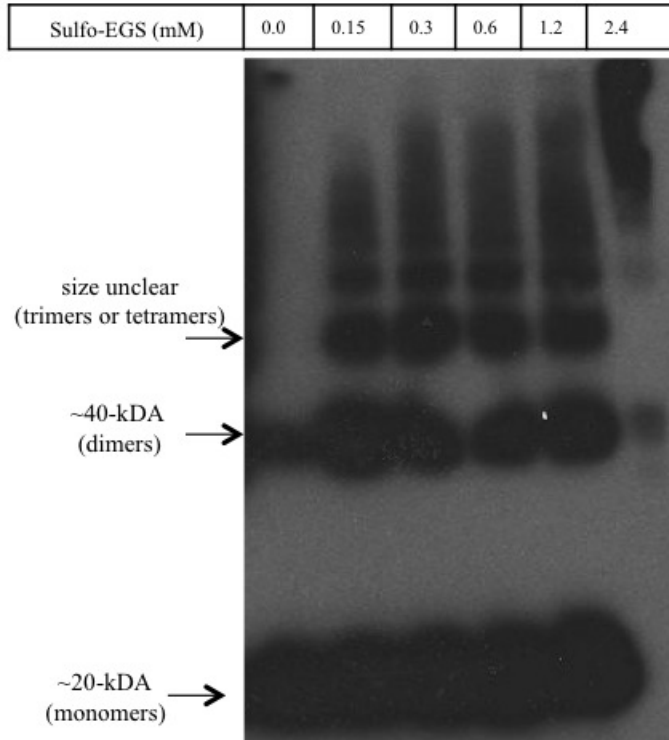


Figure 4.2 Chemical cross-linking of bacterially purified PDZD8 C-terminal fragment. BL21(DE3) *E. Coli* cells (NEB) were transformed with N-terminally HIS-FLAG-tagged PDZD8(1017-1154) in the pet22b vector (Novagen). Protein expression was induced for 4 hours with 1 mM IPTG before lysis with B-Per reagent (Pierce)/ HIS-tagged protein was purified by Ni-affinity with a HisTrap Ni sepharose column (GE Healthcare) and eluted protein dialyzed 3 times in 1x PBS. Sulfo-EGS cross-linking reagent (Pierce) was added to 250 μ L 1 mg/ml of purified PDZ (1017-1154) to a final concentration of 0-2.4 mM as indicated and mixed for 30 minutes at room temperature. Following cross-linking, each sample was quenched with 2x SDS buffer and used for SDS-PAGE gel electrophoresis. Following electrophoresis, PDZD8(1017-1154) was detected by western Blot with anti-FLAG antibody. Estimates of band size were made by comparison to a stained protein standard. This experiment was performed three times and a representative blot is presented.

4.2.2 Cofactor recruitment

Alternately I might hypothesize that PDZD8 acts to stabilize the HIV-1 core indirectly by recruiting or blocking additional capsid-stabilizing or destabilizing factors. One plausible model is that the coiled-coil motif recognizes the viral capsid while the PDZ domain recruits additional factors. The PDZ domains of other members of the PDZ domain-containing family of proteins are responsible for well-characterized protein-protein interactions, though no binding partner of PDZD8's PDZ domain has yet been identified.

I would suggest that if there were an additional stabilizing cofactor recruited by PDZD8, then that factor has not been previously identified as a HIV-1 capsid-stabilizing factor. Prior to the identification of PDZD8, the only cellular factor known to enhance HIV-1 infectivity and stabilize the viral core was CypA (23). However, our research suggests that CypA does not contribute to PDZD8-dependent capsid stabilization. Removal of cyclophilin A by centrifugal filtration does not diminish the stabilization of CA-NC tubes by lysate (Figure 2.7A).

Recent evidence has demonstrated that the cellular factor CPSF6 may also stabilize HIV-1 capsid when cytoplasmically localized (10, 20). However, I do not anticipate that this factor contributes to PDZD8-dependent stabilization. CPSF6 is normally localized in the cellular nucleus and is likely not abundant in the cytoplasmic lysates tested in the *in vitro* assays. Additionally, CPSF6 stabilization of the capsid decreases HIV-1 infectivity *in vivo* (10), while PDZD8-dependent stabilization contributes to viral infectivity. Additionally, CPSF6, like CypA, binds HIV-1 CA directly; PDZD8 would not be needed to recruit either of these factors. Instead, if

PDZD8 recruits another directly capsid-stabilizing cofactor, that factor has not yet been identified as a capsid-stabilizing entity.

One promising avenue for investigation may be the relationship of PDZD8 and the cellular cytoskeleton. Many PDZ domain-containing proteins are thought to interact with components of the cytoskeleton (5, 6, 27). Several components of the cytoskeletal multimerize to form large complexes that could serve to increase stability of the viral capsid by binding multiple molecules of CA in a manner similar to the model described in section 2.1, making these components particularly promising candidates for future study.

PDZD8 has already been reported to bind one factor associated with the cytoskeleton—the ERM protein family member moesin, which acts to cross-link the cellular membrane and cytoskeletal actin and regulates turnover of some types of microtubules (24, 25). However, I do not expect that moesin is a potential PDZD8-recruited capsid stabilizing factor. Knockdown of moesin by siRNA increases HIV-1 infection levels, an opposite effect from that observed in PDZD8 knockdown, suggesting the two do not work in concert (24). Instead, I would propose that the moesin knockdown-dependent increase in infectivity might be indicative of diminished inhibition of PDZD8-CA binding. Moesin has been reported to bind the same region of PDZD8 as HIV-1 CA, the coiled-coil motif (16). I would hypothesize that moesin may compete for PDZD8 binding with CA, diminishing capsid stability and retroviral infectivity.

A plausible variant of this cofactor recruitment model hypothesizes is that PDZD8 stabilizes HIV-1 capsids *in vivo* and CA-NC complexes *in vitro* by blocking the actions of a destabilizing component of cellular lysate. It has already been shown that the

cellular factor TRIM5 α can destabilize viral cores *in vivo* and (at high concentrations of purified protein) induce disassembly of CA-NC complex *in vitro* (32, 34). Another, unidentified active uncoating factor that acts *in vitro* has also been proposed (4). PDZD8 inhibition of this active uncoating factor or of a destabilizing restriction factor like TRIM5 α might produce results consistent with those presented in the previous chapters. However, this model is difficult to reconcile with the enhanced stability of CA-NC complexes in cellular lysate relative to buffer alone (Figure 3.2A). Such a model would suggest that cellular lysate contains an unidentified non-PDZD8 core-stabilizing factor in addition to a PDZD8-sensitive core-destabilizing factor. Furthermore, in a preliminary study I have found that the defect in HIV-1 infectivity due to PDZD8 knockdown is not substantially altered in the presence of strongly restricting (rhesus) or weakly restricting (human) TRIM5 α molecules (Figure 4.2).

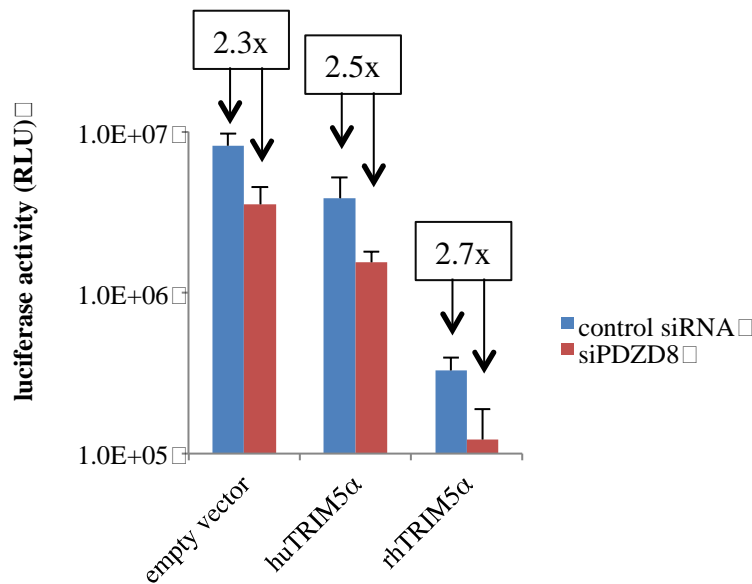


Figure 4.3 Restriction by TRIM5 α does not enhance infectivity defect from PDZD8 knockdown. Canine Cf2Th cells stably transduced with rhTRIM5 α or huTRIM5 α in pLPCX vector or empty pLPCX vector were plated to 40% confluence. Cf2Th cells were chosen because they have no endogenous TRIM5 α . 24 hours later, these cells were transfected with either an siRNA targeting canine PDZD8 or a negative control siRNA.

Figure 4.3 (continued) Forty-eight hours following siRNA transfection, cells were incubated with the indicated amount of VSV G-pseudotyped luciferase-expressing HIV-1 virus. Forty-eight hours after infection, cells were lysed and luciferase activity assayed. Above each bar, the fold-decrease in infectivity following PDZD8 knockdown is reported. The means and standard deviations from a single experiment performed in triplicate are shown. The results are indicative of results from three independent experiments.

4.3.0 Recommended Future Directions

4.3.1.0 Investigating the mechanism of HIV-1 capsid stabilization/ PDZD8 purification and lysate fractionation

I would recommend that a primary objective of future research be the experimental validation of the two models of PDZD8-dependent core stabilization I presented in section 2.0 of this chapter—*CA linking* and *cofactor recruitment*.

The most helpful experimental approach in the evaluation of these models would be an assay of CA-NC stabilization by purified PDZD8 protein. Stabilization of CA-NC tubes by purified PDZD8 protein alone would seem to invalidate the cofactor recruitment model, and indicate a greater need to focus on models of CA linking.. Alternately, should purified PDZD8 fail to stabilize CA-NC, fractionated cell lysate could be added to the purified PDZD8 to isolate additional factors required for CA-NC stabilization. It would be interesting to determine if *E.coli* lysate, which does not stabilize, enhances or inhibits stabilization by purified PDZD8.

I have previously attempted to produce HIS-tagged PDZD8 in an *E. coli* bacterial expression system. Unfortunately I was unsuccessful in producing detectable quantities of full-length PDZD8, potentially due to the large size of the factor. I was able to purify smaller HIS-tagged truncation constructs containing the C-terminal region (aa 891-1154, 932-1154, and 1017-1154). These smaller constructs did not stabilize CA-NC complexes

in vitro, unsurprising given the absence of the PDZ domain in each of these constructs.

In future work I would suggest alternate methods of PDZD8 purification including expression in insect cells as well as precipitation of tagged-PDZD8 protein expressed in mammalian cells.

Should purification of PDZD8 prove technically difficult, I would recommend an expansion of the lysate fractionation protocols discussed in chapter 2 (Figure 2.7). In particular, it may be possible to isolate any capsid-stabilizing or capsid-destabilizing cofactors of PDZD8 through additional fractionation of the lysate. One of the shortcomings of the fractionation research presented in chapter 2 is that those experiments preceded the identification of PDZD8 as primary determinant of stabilization. For that reason, lysate fractionation protocols were not optimized to isolate PDZD8 from other components of cytoplasmic lysate that may act as cofactors of stabilization. Future fractionation protocols may use knowledge of specific properties of PDZD8 (*e.g.* exact size or isoelectric point) to more efficiently isolate increasingly pure PDZD8 containing fractions.

Additionally, it may be useful to repeat previous fractionation protocols (*e.g.* size-exclusion chromatography) and assess the concentration of PDZD8 protein in stabilizing fractions. Lysate fractions containing proteins or complexes with sizes estimated to be 75-125-kDa, ~300-kDa, and >600-kDa all contributed to stabilization of CA-NC complexes (Figure 2.7A). These size ranges are generally consistent with that of PDZD8 and larger PDZD8-containing complexes (potentially PDZD8 dimers and multimers). However, as PDZD8 content was not verified, I cannot confirm that all PDZD8 fractions stabilize CA-NC complexes. The identification of PDZD8-containing fractions that fail to

stabilize would be strong evidence that either PDZD8 requires additional factors to stabilize or PDZD8's stabilization function may be deactivated (potentially by methods such as post-translational modification or binding to inhibitory factors). Any such PDZD8-tracking protocol would need to be performed with lysate treated with siRNA silencing of endogenous PDZD8 and expressing FLAG-tagged or otherwise labeled PDZD8 constructs, as no PDZD8-targeting antibody that I have evaluated has proven capable of identifying endogenous PDZD8 by western blot.

The lysate fractionalization experiments of chapter 2 also were performed before the development of the more sensitive "sucrose fractionation" variant of the tube stabilization assay (Figure 3.1). This variant of the assay is both more sensitive and quantitative, allowing better comparison of multiple stabilizing conditions. In my previous size-exclusion fractionation experiments, I consistently observed the strongest stabilization by fractions containing complexes >600-kDa. This may indicate that PDZD8 in cytoplasmic fractions is primarily present in larger complexes, or that PDZD8 in larger complexes has a greater stabilization function. To test whether larger PDZD8-containing complexes are more effective CA-NC stabilizers, I would recommend that these sensitive sucrose-fractionation stabilization assays be performed with lysate fractionated by size exclusion and then normalized by PDZD8 concentration.

4.3.1.1 Characterization of PDZD8 multimerization and structure

The PDZD8 protein itself has been the subject of minimal investigation. Analysis of PDZD8 structure would provide contextual information that would be valuable in the study of PDZD8's role in retroviral infection. Preliminary cross-linking experiments and

sequence analysis indicate that PDZD8 has a coiled-coil motif that may contribute to dimerization or higher-order multimerization, which I hypothesize may be required for core stabilization. Further investigation of PDZD8 multimerization may provide important insights into the mechanism of capsid stabilization. I have already demonstrated that peptides containing PDZD8's putative coiled-coil motif can be easily purified and that under cross-linking conditions, these peptides form dimers and higher-order complexes. These purified constructs would be ideal candidates for structural analysis (*e.g.* by X-ray crystallography) to determine the orientation of PDZD8 molecules in dimers and the role of individual amino acids in multimerization. Going forward, it would be useful to ascertain if those individual amino acids necessary for maintaining coil multimerization are also required for retroviral CA binding or capsid stabilization.

Similarly our investigation would benefit from additional analysis of the multimerization and structure of full-length PDZD8 protein. Of particular interest is whether the PDZ domain necessary for capsid stabilization contributes to higher-order association of PDZD8, a finding that would bolster the model of capsid stabilization presented in section 2.1. Structural analysis of PDZD8 by X-ray crystallography or cryo-EM imaging would be of significant value, but may be more difficult to achieve given the challenges of purifying large quantities of full-length PDZD8. Preliminary research has indicated that PDZD8 forms large complexes under cross-linking conditions, even in the absence of the coiled-coil motif. I would hypothesize that multiple regions of the PDZD8 complex may contribute to the formation of higher-order multimers required for stabilization of retroviral capsids, a model similar to that of TRIM5 α -dependent capsid destabilization. TRIM5 α higher-order multimerization has been investigated by multiple

methods, including analytical centrifugation to determine multimerization state and co-IP of tagged truncated TRIM5 α proteins to determine which regions are necessary for higher-order self-association (18, 22). I would suggest that these same methods be applied to the investigation of PDZD8 multimerization.

4.3.2 Mutational analysis of *PDZD8*

In chapter 3, I present evidence generated with PDZD8 truncation and point mutations that the putative PDZ domain and coiled-coil motif of PDZD8 are required for stabilization activity (Figure 3.5, 3.6). I would propose that this experimental approach be expanded to include additional PDZD8 constructs. The region linking the putative PDZ domain and coiled-coil motif is of particular interest. Deletion of this entire region, but not deletion of the PKC1 domain alone, abrogates stabilization (Figure 3.8). Moving forward, it would be useful to determine if specific residues in this region are required or whether inclusion of a larger linking peptide may preserve CA-NC stabilization. Additionally I would recommend expanded *in vitro* CA-NC stabilization assay and *in vivo* infectivity assay testing of single-site alanine replacement mutants of *PDZD8*. Though I have demonstrated that truncated PDZD8 constructs lacking either the PDZ domain or the coiled-coil motif fail to stabilize HIV-1 capsid, the specific residues required are still unknown. Surprisingly the PDZ peptide-binding motif does not appear critical to the stabilization function (Figure 3.7). Increased testing of alanine replacement mutants may reveal which regions of the PDZ domain are important to this function and suggest specific mechanisms.

I would also recommend alanine scanning of the putative coiled-coil, with a particular focus on isolating those residues that contribute to CA-NC binding. The PDZD8 coiled-coil motif appears necessary for binding of CA-NC and potentially critical for PDZD8 multimerization. It would be informative to determine if these two functions are separable and to identify specific residues responsible for each.

4.3.3 Expanded analysis of retroviral binding to PDZD8

In chapter 3, I present evidence that PDZD8 knockdown by siRNA decreases susceptibility of target cells to multiple retroviruses, but not Rous sarcoma virus (Figure 3.4). It is not yet understood why RSV is insensitive to PDZD8 knockdown, but one potential hypothesis is that PDZD8 does not bind the RSV capsid. RSV CA-NC forms multimeric CA-NC tubes similar to those of HIV-1 CA-NC, and may be used to assay PDZD8 binding by the protocol described in chapter 3 (9). Similarly, I would recommend assaying PDZD8 binding to tubes of additional retroviruses and alanine scanning mutants of HIV-1 *gag*. Though I might hypothesize that PDZD8 binding affinity for CA-NC will closely mirror stabilization activity in most cases, the identification of retroviral capsids that bind PDZD8 but are not stabilized by it would provide an excellent opportunity to begin future investigations into the mechanism of stabilization. An expanded analysis of PDZD8-CA binding might also include investigation of the stoichiometry of PDZD8-CA-NC binding required for stabilization.

4.3.4 PDZD8 expression analysis and knockout

Because PDZD8 has been so minimally studied in the past, I have little information regarding PDZD8 expression levels. Information of this type would provide valuable context for the study of retroviral capsid stabilization. It would be enlightening to have data regarding the expression of PDZD8 across multiple tissue types and immortalized cell lines. In my research, I evaluated HIV-1 infectivity in multiple cell lines treated with PDZD8-targeting siRNA. I found PDZD8 sensitivity differed significantly amongst these tested cell lines, and hypothesize that this sensitivity may be related to the steady-state level of PDZD8 protein in each cell line. I have not found a PDZD8-targeting antibody that is capable of PDZD8 protein level quantitation; however, RNA transcript levels may provide a suitable alternative measurement. Additionally, I would recommend the creation of a cell line with no detectable PDZD8 expression, potentially by zinc finger nuclease knockout (12). Elimination of endogenous PDZD8 expression would be useful for future mechanistic studies and may provide insight into the role of PDZD8 in uninfected cells.

4.3.5 Study of additional regulators of capsid stability by *in vitro* CA-NC assay

Finally, I believe that the *in vitro* CA-NC stabilization assay may prove to be of continuing value in the study of additional cellular factors that regulate retroviral core uncoating. Alternate *in vitro* assays of core stability have shown only limited capability to mirror the effect of cellular cofactors and restriction factors on retroviral cores *in vivo*. The *in vitro* tube stabilization assay described here has shown excellent correlation with *in vivo* assays of infectivity over a panel of PDZD8 constructs (Figure 3.10). I believe the sensitive and quantitative sucrose-fractionation variant of this assay might allow for

novel experimental approaches to the investigation of these non-PDZD8 capsid stabilizing and destabilizing factors. *In vitro*, it is possible to isolate these factors from other elements of the cytoplasmic context in ways that are not possible *in vivo*, removing small molecules such as ATP or larger protein complexes including ubiquitin ligase components. Evaluation of capsid stabilizing or destabilizing proteins in these conditions may provide important insight into the mechanistic underpinning of these processes.

Additionally, the assay may be used to identify more capsid-stabilizing cofactors. In our research, I observed that lysate from cells treated with PDZD8 targeting siRNA stabilized CA-NC complexes far more efficiently than PBS buffer alone. This may be indicative of the presence of residual PDZD8 protein or the existence of other stabilizing components. The development of PDZD8 knockout cell lines (see section 3.4) would be a valuable tool in the identification of any such novel stabilizing factors.

4.4 References

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